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EXOSOMES -
IMMUNOMODULATORS IN CANCER AND THERAPY

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Exosomes – Immunomodulators in Cancer and Therapy

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To my family

Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.

Louis Pasteur

What we observe is not nature itself, but nature exposed to our method of questioning.

Werner Heisenberg

ABSTRACT

Exosomes are nano-sized membrane vesicles derived from the late endosomal compartment. They are capable of transferring proteins, lipids and RNA between cells. B cell and dendritic cell (DC)-derived exosomes express major histocompatibility complex (MHC) class I and II, as well as costimulatory molecules (CD80/86) and can initiate T cell responses. Several clinical trials have shown DC-derived exosome-based cancer immune therapy to be safe but limited in inducing antigen-specific T cells. In contrast, tumour cell-derived exosomes can express immune inhibitory molecules and play an important role in spreading oncogenic activity by carrying tumour antigens, inducing angiogenesis at distant sites and preparing tissues for metastasis. This thesis aimed at I) analysing how to enhance the immunogenicity of exosomes for therapy, II) investigating whether MHC complexes on exosomes are needed to induce an anti-tumour immune response, III) comparing microvesicles and exosomes side by side for their immunogenic capacity, IV) understanding the metastatic process induced by tumour-derived exosomes from bladder cancer patients and whether certain exosomal proteins can be used as markers for diagnosis and prognosis.

Study I reveals that exosomes loaded with the NKT cell ligand alpha-galactosylceramide (αGC) and the model antigen ovalbumin (OVA) activate NKT cells, induce strong NK and γδ T cell innate immune responses, and induce OVA-specific T and B cell responses far better than only OVA-loaded exosomes. Exosomes loaded with αGC/OVA decreased tumour growth and increased median survival compared to exosomes loaded with OVA only or soluble αGC + OVA alone in a B16 melanoma model. This study demonstrates how to increase the immunogenicity of DC-derived exosomes for cancer treatment.

Study II demonstrates that exosomal MHC class I is dispensable for the induction of antigen-specific T cell responses if whole OVA is present. We show that OVA-loaded DC-derived exosomes from $MHCI^{-/-}$ mice induce antigen-specific T cells to the same extent as wild type exosomes. Even exosomes with MHC class I and II mismatch induced tumour-infiltrating $CD8^+$ T cells and increase survival in a B16 melanoma model. This study provides new opportunities for the design of allogeneic exosome-based vaccines and therapies.

Study III compares microvesicles (MV) and exosomes from OVA-exposed DCs side by side for their capacity to induce OVA-specific immune responses *in vivo*. MV and exosomes express similar surface markers but only exosomes induced OVA-specific $CD8^+$ T cells and OVA-specific IgG antibodies. In contrast, MV induced a higher number of plasma cells. Finally, we found that exosomes contain more OVA compared to MV. We conclude that exosomes from DCs are superior in inducing antigen-specific immune responses *in vivo* compared to MVs, while MVs might activate the immune system unspecifically.

Study IV evaluates the proteomic profile of exosomes from tumour tissue explants and urine from urinary bladder cancer patients. We show that exosomes from malignant or benign tissue can be distinguished by the proteomic profile and are involved in platelet, metabolic and immune signalling networks. We show that, even if no tumour is left, exosomes can express a metastatic memory phenotype which might be involved in cancer progression.

In summary, this thesis gives new insights into how to design vesicle-based cancer vaccines and provide new opportunities for the use of allogeneic DC-derived exosomes in patients. In addition, we demonstrate that exosomes isolated from the urine of urinary bladder cancer patients express specific markers for malignancy, which provides new possibilities for diagnostic strategies.

LIST OF SCIENTIFIC PAPERS

I. Gehrmann U, **Hiltbrunner S**, Georgoudaki AM, Karlsson MC, Näslund TI, Gabrielsson S. **Synergistic induction of adaptive antitumour immunity by codelivery of antigen with α-galactosylceramide on exosomes**. Cancer Research, 2013, Jul 1;73(13):3865-76

II. **Hiltbrunner S***, Larssen P*, Eldh M, Martinez Bravo MJ, Wagner AK, Karlsson MC, Gabrielsson S. **Exosomal cancer immunotherapy is independent of MHC complexes on exosomes**. Oncotarget, 2016, May 25

III. Wahlund CJE, **Hiltbrunner S**, Näslund TI, Gabrielsson S. **Exosomes from antigen-pulsed dendritic cells induce stronger antigen-specific immune responses than microvesicles *in vivo***. in manuscript

IV. **Hiltbrunner S***, Mints M*, Eldh M, Rosenblatt R, Holmström B, Alamdari F, Johansson M, Hansson J, Vasko J, Winqvist O, Sherif A, Gabrielsson S. **Exosomes reveal a metastatic memory profile in patients with invasive urothelial bladder cancer**. in manuscript

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SUPPORTING REVIEWS

Bell BM, Kirk ID, **Hiltbrunner S**, Gabrielsson S, Bultema JJ, **Designer exosomes as next-generation cancer immunotherapy**. Nanomedicine, 2016 Jan;12(1):163-9

Gehrmann U, Näslund TI, **Hiltbrunner S**, Larssen P, Gabrielsson S. **Harnessing the exosome-induced immune response for cancer immunotherapy**. Semin Cancer Biol. 2014, Oct;28:58-67

Gehrmann U., **Hiltbrunner S**, Näslund T, Gabrielsson S. **Potentiating antitumour immunity with αGC-loaded exosomes**. OncoImmunology, 2013, Oct 1;2(10):e26261

CONTENTS

LIST OF ABBREVIATIONS

αGC	alpha-galactosylceramide
Ab	Antibody
ACT	Adoptive cellular therapy
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cells
BrdU	5-bromo-2'-deoxyuridine
DC	Dendritic cell
EM	Electron microscopy
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
Exo-OVA/αGC	OVA and αGC loaded exosomes (paper II)
Exo-(αGC-OVA)	OVA and αGC loaded exosomes (paper I)
EV	Extracellular vesicles
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
i.d.	Intradermal
ILV	Intraluminal vesicle
IFN	Interferon
i.v.	Intravenous
LPS	Lipopolysaccharid
MAGE	Melanoma-associated antigen
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIBC	Muscle invasive bladder cancer
MMM	Marginal zone metallophilic macrophages
MZM	Marginal zone macrophages
MV	Microvesicle
MVB	Multivesicular body
NAC	Neoadjuvant chemotherapy
NKT	Natural killer T cells
NMIBC	Non-muscle invasive bladder cancer

OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PCa	Prostate cancer
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RNA	Ribonucleic acid
s.c.	Subcutaneous
SIINFEKL	Major ovalbumin peptide for H2Kb
snMase	Neutral spingomyelinase
TAA	Tumour-associated antigen
TCR	T cell receptor
TEX	Tumour-derived exosomes
Tfh	T follicular helper cells
TIL	Tumour-infiltrating lymphocytes
Treg	Regulatory T cells
TUR-B	Transurethral resection
UBC	Urinary bladder cancer
VLP	Virus-like particle

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM IN CANCER

1.1.1 Historical Overview of Tumour Immunology

The earliest reference of immunity is from 430 BC when a Greek historian in Athens described a plague outbreak. He described that people who survived the illness can nurse infected people without suffering from the infection a second time. Interestingly, time passed until the concept of immunity was described a second time. In the 15th century, the Chinese prevented smallpox infections by using dried crusts from smallpox pustules as an inhalation vaccine or put small pieces into cuts in the skin (variolation). This was a huge success in prevention of new infections; however, severe and fatal reactions after variolation were occurring [1].

In 1798 Edward Jenner made an important discovery; he realized that milkmaids who were in contact with cows suffering from cowpox were immune against severe smallpox infections. He injected an eight-year old boy with the fluid from a cowpox pustule and infected the boy later on with smallpox. Surprisingly and fortunately, the boy did not become sick. In the summer of 1881, Louis Pasteur noticed that cholera bacteria, from an old culture forgotten over summer, gave chickens only minor symptoms and more importantly protected them from getting infected a second time with a fresh stock of cholera bacteria. He described the concept of vaccination and further developed his findings for other diseases and used weakened or attenuated strains as a vaccine. This is considered the beginning of modern immunology [2]. In 1883 Elie Metchnikoff described a white blood cell, which was able to take up microorganisms or other small particles. He named it phagocyte and discovered the cellular part of the immune system. During the same time in the late 19th century, von Behring and Kitasato discovered that the serum from animals previously infected with diphtheria could transfer immunity to unimmunized animals. They discovered the humoral part of the immune system.

Interestingly, already in 1891 a surgeon treated his cancer patients with bacterial products, which induced inflammation and led to a reduction of the tumour mass. However, for a long time engagement of the immune system to fight against cancer was doubted due to poor results [3]. In the 1930s Elvin Kabat showed that the transfer of immunity was mediated by gamma globulins (today immunoglobulins). Hence, the concept of humoral immunity was born and led to many new treatment options and the term passive immunity was established. Vaccines, on the other hand, are referred to as active immunity, engaging the immune system´s possibility to react to an antigen and to develop immunity. In the late 1940s the first chemotherapy was approved by the US food and drug administration (FDA). This was the time when the understanding of the involvement of the immune system in cancer grew. Edward J. Foley discovered that inbred mice transplanted with a tumour, followed by tumour removal, were more resistant to a second transplantation. In 1959, the tuberculosis vaccine

Bacillus Calmette-Guérin (BCG) inhibited tumour growth in mice. BGC installation is, still up to this date, a potent therapy in non-invasive superficial bladder cancer.

Today, researchers are pursuing several lines of immunology-based cancer therapeutics including vaccines, check point inhibitors, depleting antibodies and chimeric antigen receptor (CAR) T cells. In addition, they are still trying to understand how the body's own immune system can be used to fight cancer, how tumour cells influence the tumour microenvironment and how metastasis develop at distant sites. The understanding of how to trigger a potent cellular memory and antibody response is a big challenge in the development of anti-cancer immunotherapies and cancer vaccines. The development and identification of new adjuvants (e.g. nanoparticles) and the use of neoantigens to induce specific anti-tumour immune response are ongoing.

1.1.2 Cancer and the Immune System

For a long time engagement of the immune system to fight cancer was doubted due to poor results in several clinical trials. The field changed mid-1990s when it was shown that transplanted tumours grow better in mice treated with a neutralizing antibody against IFNγ [4] and mice lacking a functional IFNγ pathway or T cell compartment were more susceptible to chemically induced sarcomas [5, 6]. This was the first time shown that the immune system plays a crucial role in controlling tumour growth. In addition, the immune system also defines the characteristics of the tumour. 3-methylcholanthrene (MCA)-induced tumours grown in $Rag2^{-/-}$ mice and transplanted into naïve syngeneic mice led to tumour eradiation. In contrast, MCA-induced sarcomas grown in immunocompetent syngeneic mice grew progressively when transplanted into naïve syngeneic mice [7]. These results demonstrate that tumours are immunogenic and induce an anti-tumour immune response, which can eliminate cancer cells. Thus, the concept of immunoediting was developed. Cancer immunoediting is a dynamic process, which consists of three different phases, the elimination phase, equilibrium phase and escape phase.

Elimination phase

The elimination phase is characterized by the recognition of transformed tumour cells by the innate and adaptive immune system. Mice lacking NKT cells ($CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice), NK cells or αβ T and γδ T cells are more susceptible to carcinogen induced tumours [8-10]. The tumour microenvironment during the elimination phase is pro-inflammatory and characterized by expression of interferons. IFNγ can on one hand upregulate MHC class I expression of tumour cells, leading to recognition by $CD8^+$ T cells or activate the host´s immune system to fight cancer [7]. Mice lacking parts of the cytotoxic pathway like perforin, TRAIL or FasL are also more susceptible to age induced tumours [11-13].

Equilibrium

Many tumours are poorly immunogenic and the balance between cancer cell proliferation and destruction is called equilibrium phase. This phase is mainly mediated by the adaptive immune system. Koebel and colleagues showed in 2007 that cancer lesions induced by low doses of carcinogens, rapidly grew out once the $CD4^+$ and $CD8^+$ cells and/or IFNγ were depleted. However, this was not the case when depleting NK cells or NK cell function (using anti-NKG2D, anti-TRAIL antibodies) [14].

Escape phase

In the escape phase the immune system has failed to eradicate tumour cells and they can divide rapidly and acquire different genetic alterations. The tumour microenvironment changes to be immunosuppressive, mediated by regulatory T cells, myeloid-derived suppressor cells (MDSC) and tumour-associated macrophages (TAM), all inhibiting T cell activation. There are different immune escape strategies, (I) tumour cells can be rendered invisible to the tumouricidal $CD8^+$ T cells if they do not express neoantigens or downregulate specific tumour-associated antigens (TAA). (II) Furthermore, cancer cells secret soluble factors, which can influence the tumour-associated immune cells. They can secrete soluble NKG2D ligands, which block activating NK cell receptors [15], or vascular endothelial growth factor (VEGF), which leads to enhanced angiogenesis and inhibits the maturation status of DCs [16]. Tumour cells can also secrete IL-10 or TGFβ, which act as inhibitory cytokines, promote differentiation of regulatory T cells and skew the immune system towards a Th2 phenotype [17]. The expression of the enzyme IDO by tumour cells and antigen presenting cells leads to production of immunoinhibitory metabolites, which induce T cell anergy and apoptosis [18]. (III) Tumour cells can also influence the immune system by downregulating molecules involved in T cell recognition (MHC class I, β2m) [19, 20] or express mutated forms of certain death receptors [21]. Interestingly, tumour cells can also express immune inhibitory ligands like PD-L1, which leads to dampening of cytotoxic T cell responses or apoptosis of T cells [22]. This whole orchestra of suppressive cells and cytokines leads to the failure of anti-tumour immunity and to cancer progression.

1.1.3 Dendritic Cells

1.1.3.1 General overview

Dendritic cells (DCs) are bridging the innate with the adaptive immune system by recognizing antigens and inducing antigen-specific immune responses. DCs have been shown to be the most efficient antigen presenting cells in stimulating naïve T cells [23]. They are specialized in the uptake and processing of antigens in order to present peptides on MHC complexes to T cells [24]. DCs are located at pathogen exposed sites such as mucosal surfaces, where they constantly sample the environment for pathogens. After they have encountered a pathogen/antigen they differentiate into a mature phenotype, upregulate CCR7,

MHC class I and II and costimulatory molecules [23] and migrate to the regional lymph node to activate T cells.

DCs take up antigens through different mechanisms such as receptor-mediated endocytosis (mainly lectin receptors recognizing carbohydrates), phagocytosis and macropinocytosis. After uptake, proteins are digested in the phago-lysosome and peptides are loaded onto MHC class II molecules in the endocytic pathway for presentation to $CD4^+$ T cells. On the other hand, MHC class I destined peptides are produced in the cytosol by degradation of endogenous proteins or foreign antigens by the proteasome. These peptides are transported via the TAP transporter into the lumen of the rough endoplasmatic reticulum (rER) where they can bind MHC class I complexes (Figure 1). However, also exogenous antigens can be loaded onto MHC class I molecules by a process called cross-presentation. Two pathways are suggested for cross-presentation. In the cytosolic pathway antigens are transferred from the endosome into the cytosol. DCs, compared to macrophages, have a relatively mild endosome and low levels of lysosomal proteases, therefore intact antigens can escape the endosome. In the cytosol, they are degraded by the proteasome and are transferred into the ER through the TAP transporter to be loaded on MHC class I molecules [25, 26]. In the vacuolar pathway antigens are directly degraded in the endocytic compartment and loaded onto MHC class I molecules [27].

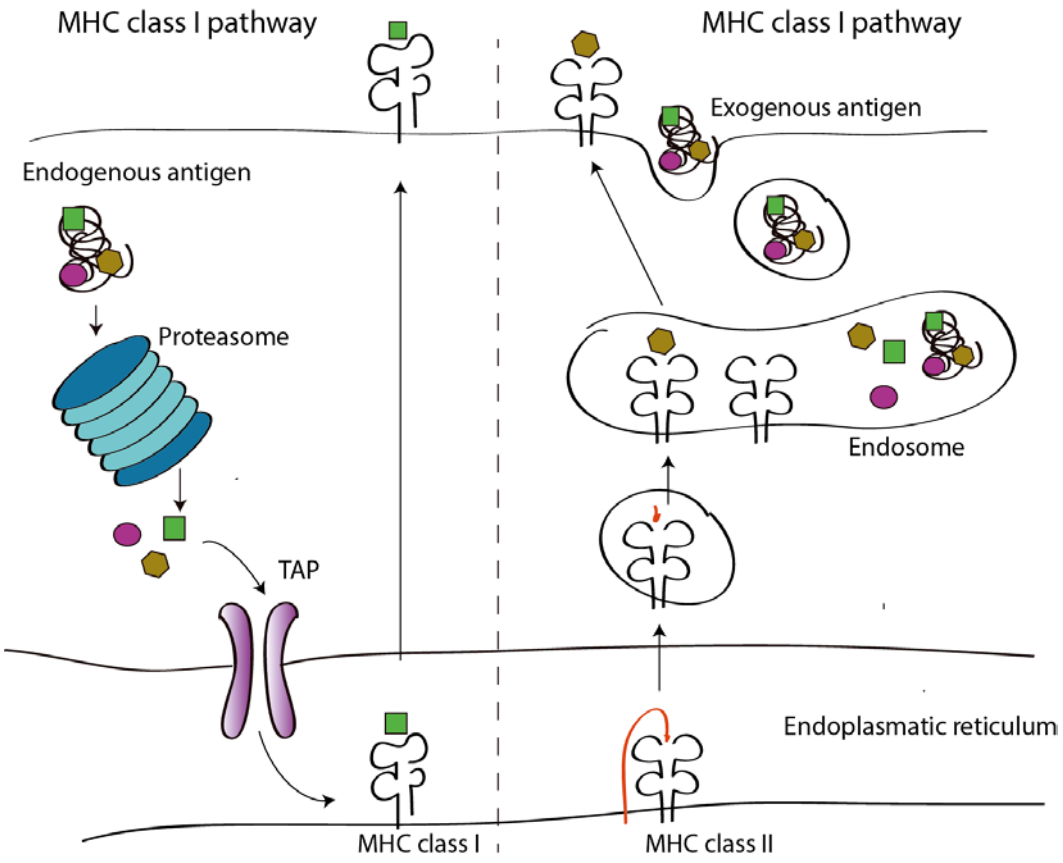

Figure 1: Processing of endogenous and exogenous antigens by dendritic cells and loading of MHC class I and II molecules.

DCs are a heterogeneous group of cells and differ in their capacity to regulate Th1 or Th2 immunity. Certain subsets can induce high levels of type I interferons or cross-present antigens to $CD8^+$ T cells. Depending on the exogenous stimuli and subset, DCs will respond differently by producing different cytokines or upregulating certain genes [28-30]. For example, virus-like particles from human papilloma virus induce IFNγ expression in $CD8\alpha^+$ DCs but Th2 cytokines in $CD8\alpha^-CD11b^+CD11c^+$ DCs [31]. LPS and inflammatory cytokines such as TNFα or CD40 ligand can induce DC maturation, which leads to expression of molecules involved in T cell activation [32, 33]. DCs activated through TLR ligands are able to produce IL-12 and can activate other DCs, which have not been stimulated by a TLR ligand. However, these DCs fail to produce IL-12 but upregulate MHC and costimulatory molecules and can activate naïve T cells in the same manner as directly activated by TLR ligands. Interestingly, T cells activated by indirectly activated DC cannot exert Th1 effector functions [34].

1.1.3.2 Dendritic Cell Therapy in Cancer

As described above, the goal of tumour immunotherapy is to activate the patient´s own immune system to elicit a potent anti-tumour immune response. Currently, monoclonal antibody therapy against CTLA-4 in metastatic melanoma showed promising results and was approved by FDA in 2011. However, these therapies are only effective with a pre-existing anti-tumour immune response in the patient [35]. The combination of checkpoint blockade and cell therapy for the induction of tumour-specific T cells might be an attractive approach. The goal of DC therapy in cancer is on one hand to induce tumour-specific $CD4^+$ and $CD8^+$ T cells, which can regulate tumour growth and on the other hand the induction of memory T cells to prevent relapses. Many clinical trials using DC therapy were shown to be safe with low side effects and no immune related toxicity but with suboptimal clinical success [36]. In contrast, checkpoint blockade antibodies can induce severe immune related side effects and autoimmunity [37]. DC vaccination induces tumour-specific T cells in many patients [38], however, the clinical efficacy is still very limited, even though there are patients with complete remission. Thus, DC vaccination needs further refinement to increase the frequencies of responders, possibly by finding biomarkers for personalized treatment strategies [36]. The low efficacy might be due to a strong immune suppressive tumour microenvironment, limited T cell migration into the tumour and suboptimal selection of patients, where mainly patients with advanced cancer and poor prognosis have been treated so far. Nevertheless, current treatment protocols (chemotherapy, radiotherapy) in these patients show similar clinical efficacy as DC therapy [36]. In conclusion, different simulation protocols of DCs and combination therapies need to be further investigated.

Currently, there are many different protocols used in clinical trials. Often monocyte-derived DCs cultured with IL-4 and matured with pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and prostaglandin E2 and/or TLR ligands are used in DC-based cancer therapy. The maturation status of the DC is important for the clinical outcome. Matured DC showed better results in patients with prostate cancer, melanoma and glioma [39, 40]. Interestingly, DC

matured with TNFα, IL-1β, IFNγ, IFNα and polyI:C showed good clinical activity [41]. Currently, also the use of different DC subsets, such as Langerhans-like DCs, which are very efficient in simulating cytotoxic T cells, are evaluated [42, 43]. In summary, DC-based therapies need further refinement to show good clinical efficacy and to elicit strong T cell responses. Selection of the right patient group might improve clinical responses and survival.

1.1.4 $CD8^+$ T Cell Responses

Upon antigen encounter DCs may activate $CD8^+$ and $CD4^+$ T cells. How the interaction between $CD4^+$ and $CD8^+$ and DC is occurring is still under debate. The "three cell interaction" model proposes that $CD4^+$ and $CD8^+$ T cells must bind to the same DC in order to activate the $CD8^+$ T cell to differentiate into an effector cell. However, another model the "dynamic model of sequential two-cell interactions by APC", introduced by Ridge *et al.* proposes that $CD4^+$ T cells license the DC first by CD40-CD40L interaction. In a second step the "licensed" DC can efficiently activate $CD8^+$ T cells [44].

Many groups have described that activation of DCs through CD40 to be crucial for inducing CTL responses [45]. Matzinger´s group demonstrated that activation of DCs *in vitro* by anti-CD40 antibody can activate CTL responses *in vivo* in mice deficient in helper T cells [44]. However, this model was questioned when in 2002 Tanchot and colleagues published that CD40 expression is not crucial on APC but on $CD8^+$ T cells. Activated $CD4^+$ T cells express CD40L and peptide/MHC complexes and are able to induce CTL responses directly [46]. Interestingly, DCs can transfer costimulatory molecules and peptide/MHC complexes to $CD4^+$ T cells and these $CD4^+$ T cells can activate naïve $CD8^+$ T cells *in vitro* and *in vivo* [47]. This transfer is TCR/MHC/peptide specific [48]. A study with different antigen secreting tumour cells revealed that only antigen bound to vesicles (exosomes) induced a strong CTL response and activated $CD4^+$ T cells efficiently for delivering help for $CD8^+$ T cells [49]. *In vivo* evidence showed that $CD4^+$ T cell/$CD8^+$ T cell/DC cell clusters are not important for CTL priming. The dynamic model rather suggest independent interactions between $CD4^+$ T cells and APC and licensed APC with $CD8^+$ T cell and subsequent activation of $CD8^+$ T cells by $CD4^+$ T cells [50].

$CD8^+$ T cells are in general important cells in controlling infections of intracellular pathogens such as viruses. They recognize an infected cell by its expression of MHC/peptide complexes through the TCR. In tumours, $CD8^+$ T cells recognize cells expressing aberrant peptides presented on MHC class I, which induces killing of the tumour cells either by perforin/granzymes or Fas/FasL induced apoptosis. Furthermore, they secrete IFNγ which induces upregulation of MHC class I on the target cells. Therefore, the potent induction of tumour-specific $CD8^+$ T cells by cancer vaccines is of great interest and is an important part to control tumour growth.

1.1.4.1 Antibody-dependent cellular cytotoxicity

Besides the induction of a strong $CD8^+$ T cell response to eradicate tumour cells, a category of $CD4^+$ T cells (T follicular helper cells) play a crucial role in stimulating B cells for production of high-affinity antibodies [51]. The interaction of CD40 on B cells and CD40L expressed on T cells is crucial for affinity maturation and isotype switching of antibodies as well as the formation of memory B cells [52]. Production of tumour-specific antibodies leads to specific recognition of tumour cells and to subsequent binding of NK cells, macrophages and neutrophils via Fc receptors. Thus, innate immune cells are activated and secrete cytotoxic granules containing perforin and granzymes to kill the tumour cell. This process is called antibody-dependent cellular cytotoxicity (ADCC). An important aim of cancer vaccines (e.g. DC-derived exosomes) is to enhance antibody production by B cells and further stimulate cytotoxic cells such as NK cells to induce ADCC. Interestingly, the activation of iNKT cells by αGC and the addition of TLR ligands enhanced ADCC mediated killing by NK cells *in vitro* [53]. A similar effect was seen by activating NK cells with IL-2 and IL-21 [54, 55].

1.1.5 Cancer Immunotherapy

1.1.5.1 General overview of cancer immunotherapy

The main goal of tumour immunology is to understand mechanisms involved in induction of anti-tumour immune responses and tumour rejection. Immunotherapy aims at initiating or augmenting an anti-tumour immune response to eradicate established tumours. Therefore, recognizing the tumour as “foreign”, identifying specific tumour-associated antigens (TAA) and potentiating antigen-presenting capacity are major goals of cancer immunotherapy.

A weak physiological anti-tumour immune response can have different reasons. The immune system needs to recognize mutated “self” proteins. Normally T cells with high avidity towards self are deleted due to central and peripheral tolerance. Therefore, TAA-specific T cells are not very abundant in the tumour. In addition, there are no strong innate stimuli like pathogen-associated molecular patterns (PAMPs), which kick start the immune system and the antigen concentration can be very low. Also, the antigen concentration threshold for the activation of immunosuppressive regulatory T cells is much lower compared to naïve T cells which need 10 to 100 fold higher concentration, thus impeding anti-tumour responses [56].

Currently, two types of cancer immunotherapies have shown promising results during the last decades: immune-cell-targeted monoclonal antibody (mAb) therapy and adoptive cellular therapy (ACT). In the late 90s, different antibody-based targeted cancer therapies reached the market, a monoclonal anti-CD20 antibody for treatment of non-Hodgkin-lymphoma [57] and an antibody targeting Her2/neu in metastatic breast cancer [58]. In addition, the discovery of CTLA-4 and PD-1 [59, 60] resulted in the development of checkpoint blocking antibodies [61, 62]. mAb for targeting immune checkpoint molecules are not specific for a particular cancer type, but will influence the patient’s own immune system. Blocking of immune

regulatory molecules leads to more T cell activation and to a stronger anti-tumour immune response. Anti-CTLA-4 (ipilimumab) for treatment of metastatic melanoma has been approved by FDA in 2011 and anti-PD-1 (nivolumab) in 2014 for NSCLC [62] and melanoma [61]. Immune checkpoint blockage therapy can lead to tumour regression comparable with current cytotoxic chemotherapy treatments [63, 64]. Other promising results were shown by the transfer of *ex vivo* expanded tumour-infiltrating lymphocytes (TIL) or by chimeric antigen receptor (CAR) engineered T cells. CAR T cells express variable heavy and light chains of an antibody on their surface and are able to bind specific antigens. Upon binding, the T cell becomes activated and can kill the target cell. Second generation CAR T cells contain a costimulatory domain intracellularly for signal 2 and have shown up to 90% remission rates in advanced refractory B cell malignancies when targeting CD19, present on the B cell surface [65, 66]. However, treatment with CAR T cells requires known expression of a specific protein on the cell surface, which can be targeted. Thus, the investigation of specific protein signatures on cancer cells is of great need.

Unfortunately, the efficacy of cancer vaccines, to induce a strong Th1 immune response and TAA-specific cytotoxic T cells to kill tumour cells, has been low. The list of approved adjuvants is very limited and many approved adjuvants such as alum induce a Th2 biased immune response [67]. Often specific $CD8^+$ T cells get induced at the injection site (e.g. subcutaneous) but fail to migrate into the tumour (water in oil emulsion). Therefore, new approaches of vaccine design, delivery and adjuvant technology need to be developed and engineered. Besides using the best possible adjuvant for a cancer vaccine, the presence of the right peptides/proteins is crucial for inducing an anti-tumour immune response. Many studies focused on peptide-based cancer vaccines with poor therapeutic efficacy [68]. However, as mentioned above, many tumour antigens are unknown and specific T cells are rare due to thymic selection. The engagement of a broader set of T cells by immunization with different long peptides binding to MHC class I and II molecules have shown better results in certain clinical trials [69, 70]. An alternative approach can be the use of neoantigens, which arise during cancer progression. High mutation rates lead to higher frequencies of neoantigens and TILs [71]. The mutation rate between different tumours can vary, while melanoma and bladder cancer are malignancies with a high mutation rate, thyroid cancer and acute myeloid leukemia (AML) are the opposite [72]. Neoantigens can be analysed by biopsies and further bioinformatic analysis [73] and engineered into special designed personalized vaccines [74, 75]. However, this is a very costly approach and beside the induction of tumour-specific T cells, immunoinhibitory effects in the tumour must be considered as well for effective cancer therapy.

1.1.5.2 Nanoparticles as immune adjuvants

Live attenuated viruses or inactivated pathogens are the most potent vaccines existing. However, the variation between different batches and side effects in immune compromised people can be a problem. Subunit vaccines consisting of only certain proteins are generally safer but unfortunately poorly immunogenic. Therefore, the understanding of nanoparticle-based antigen delivery vehicles could improve current vaccine design for cancer therapy.

Currently, there are different nano- and microparticle-based delivery systems in development. Virus like particle (VLP)-based vaccines such as Gardasil (Merck) and Cervarix (Glaxo Smith Kline) against human papilloma virus (HPV) induced cervical cancer are already approved and on the market [76].

The goal of a cancer vaccine is the induction of a broad spectrum of immune cells which, recognize and kill cancer cells. This includes activation of NK cells, NKT cells, $CD8^+$ T cell and activation of B cells for the production of TAA-specific antibodies and finally the induction of long-lived helper T cells. DCs are the most crucial cells to elicit a strong primary immune response. DCs are phagocytic cells and the most potent antigen presenting cells in the immune system [23]. Antigen capture by immature DC and maturation through toll like receptors (TLR) activation induce upregulation of MHC and costimulatory molecules needed for T cell activation. The uptake of particles by DCs is dependent on size, shape, surface charge and interaction with surface receptors. Nano-sized particulate antigens have a large surface, which can expose charged molecules. This can enhance interaction with DCs and can therefore lead to better uptake of the antigen, efficient MHC loading and presentation to T cells [77].

Extracellular antigens are taken up by APC, processed in the endo-lysosomal compartment and presented on MHC class II molecules to activate $CD4^+$ T cells as mentioned above. Presentation of extracellular antigens on MHC class I molecules follows a different processing pathway called cross-presentation, either through the cytosolic or the vacuolar pathway [27]. Different studies show accumulation of antigen in the cytosol, enhanced cross-presentation and induction of $CD8^+$ T cells after injection of engineered nanoparticles [78]. In addition, nanoparticles (< 200 nm) are superior in priming $CD8^+$ T cells compared to microparticles (> 1 µm) and show better efficacy in inducing anti-tumour responses [79]. Interestingly, already in 1994 it was shown that macrophages take up antigen bound to latex beads and present it on MHC class I molecules 100 to 1000 times more efficiently compared to soluble antigens [80]. Particles with a diameter of 40 to 100 nm are efficiently taken up by $DEC205^+$ DCs, however, larger particles predominantly bind to $F4/80^+$ macrophages and could be taken up by phagocytosis or pinocytosis [79]. Furthermore, it has been reported that smaller sized particles (around 200 to 600 nm) induced a stronger cellular immune response compared to bigger particles (> 2 µm), which induced a better antibody response [81, 82]. Nanoparticles are inducing a more Th1 prone immune responses compared to microparticles, which favour a Th2 response [82, 83]. However, other authors claim that the particle size is not influencing the Th1/Th2 immune response [84]. The discrepancy of results may be due to different injection routes, particle composition and structure and different animal models used in the studies. Interestingly, also exosomal antigens have been shown to be cross-presented on APC in similar levels as antigen loaded VLP [85-87].

Small particles subcutaneously injected passively drain to the draining lymph node, whereas larger particle from 500 to 1000 nm can be trapped at the injection site and need to be taken up by a DC to be transported to the lymph node [88]. Nanoparticles can carry native antigens

and bind directly to the B cells in the follicle. In addition, particulate antigens are also able to crosslink the BCR on B cells which leads to direct activation of antigen-specific B cells independently of T cell help [89]. A major advantage of using nanoparticles as vaccines is the possibility to engineer them to deliver several TLR ligands and antigens on the same particle. Recently, Mandraju and colleagues described that the combination of different TLR ligands enhance or inhibit $CD8^+$ T cell activation; TLR3, TLR7 and TLR9 ligands are favouring $CD8^+$ T cell induction [90]. CpG has been widely studied in cancer vaccines and shows a strong $CD8^+$ T cell induction when loaded onto exosomes [91]. Exosomes engineered to carry different TLR ligands and certain antigens might be a perfect tool to target specific immune cells and to elicit a strong anti-tumour response. In conclusion, nanoparticle-based vaccines might mimic the nature and the structure of viruses and promote both humoral and cellular immune responses.

1.2 INITIATION OF AN IMMUNE RESPONSE IN THE SPLEEN

The spleen is the essential organ for capturing blood-borne antigens. The blood passes through the spleen with a low flow rate enabling many specific immune cells to capture antigens from the blood directly and to induce an immune response. The blood is entering the spleen through the central arterioles and flows through the marginal sinus where the antigens can be captured and transferred to the B cell follicles. Marginal zone metallophilic macrophages (MMM) are located at the inner site of the marginal sinus, close to the white pulp. Marginal zone macrophages (MZM) on the other hand are located at the outer site of the sinus. Both macrophage types express different pattern recognition receptors (PRR) like scavenger receptors or C-type lectin receptors to capture antigens [92]. In addition, DCs in the circulation can capture antigens and transport them directly to the marginal sinus. CD169 (Siglec-1) expressing MMM are found in the B cell zone after LPS stimulation [93], which indicates that they might play a role in antigen transfer from the marginal zone to the B cell area. Furthermore, antigens bound to $CD169^+$ MMM are transferred to $CD8^+$ DCs leading to cross-presentation and subsequently to the activation of $CD8^+$ T cells [94]. $CD8^+$ DCs are located in the T cell zone and outer marginal zone, $CD8^-$ DCs can be found in the red pulp and marginal zone. However, $CD8^+$ and $CD8^-$ DCs capture similar amounts of soluble antigen or antigen coated beads, but only $CD8^+$ DC are able to cross-present these antigens [95]. Therefore, $CD8^+$ DCs need a specialized machinery for cross-presentation [96]. Interestingly, $CD8^+$ DCs express a specific set of proteins involved in MHC class I presentation including TAP1 and TAP2. In contrast, $CD8^-$ DCs express proteins involved in MHC class II presentation [97]. DCs can also take up antigens via FcγRIIB, which leads to the access of a non-degradable pathway in DCs and to recycling of the native antigen on the cell surface and subsequent activation of B cells via the BCR [98].

Marginal zone B cells (MZB) are located in the outer layer of the marginal sinus and express sphingosine-1-phosphate (S1P) receptors 1/3, which bind S1P from the blood. This signal retains the MZB cells in the marginal zone and interferes with the strong attraction signal towards the follicle expressed by follicular dendritic cells (FDC). In addition, MZB cells

interact with stromal cells through the expression of integrins (αLβ2 or LFA1) with ICAM and VCAM on stromal cells to retain the MZB cell. MZB express poly-reactive BCRs, which recognize different microbiological patterns and clear bacteria from the blood. Binding of blood-borne microorganisms to the BCR of the MZB and simultaneous engagement of TLR leads to the production of low affinity antibodies [99]. This T cell independent pathway for the production of low-affinity IgM antibodies is important for the first line of defence until follicular B cells take over with the production of high-affinity antibodies. As mentioned above, MZB could also encounter antigens from MMM or MZM or from DC and neutrophils captured in the periphery. DCs provide survival signals to MZB through the expression of BAFF and APRIL [100] by binding to the transmembrane activator and CAML interactor (TACI) on MZB. This induces a signal to MZB for class switch recombination and antibody production.

Upon binding of antigens to MZB by complement receptors CD21 or CD35, MZB downregulate S1P receptors and upregulate the chemokine receptor CXCR5. Thus, they migrate to the follicle and can deposit the antigen on the FDC, subsequently they downregulate the chemokine receptor and can migrate back to the marginal zone. On the other hand, MZB can migrate to the PALS, where they initiate a germinal center (GC) response by binding to cognate $CD4^+$ T follicular helper cells (Tfh), which have been activated by DCs. The antigen on the FDC leads to selection of high affinity germinal center B cells through somatic hypermutation. The interaction of GC B cells with Tfh cells through CD40-CD40L and MHC-TCR binding, leads to the development to plasma cells and long lived memory cells with high-affinity IgG production.

1.3 NKT CELLS

1.3.1 General Overview

Natural Killer T (NKT) cells are bridging the innate and adaptive immune system and express receptors which are characteristic for NK cells and T cells. They recognize glycolipid antigens by a restricted set of TCR presented by the non-classical MHC class I like molecule CD1d [101]. Compared to MHC class I molecules, which are expressed by all nucleated cells, CD1d is only expressed by DCs, macrophages and B cells, at high levels by marginal zone B cells [102]. NKT cells express the NK cell marker NK1.1 in mice and a restricted set of TCRα (mice Vα14-Jα18, humans Vα24-Jα18) and TCRβ (Vβ8, Vβ7, Vβ2 in mice, Vβ11 I humans) chains. This goes in line with the fact that Vα14 TCR transgenic mice have higher percentage of NKT cells [103] and $J\alpha18^{-/-}$ mice lack NKT cells completely [104]. NKT cells also express T cell markers such as CD25, CD44 and CD69 and the majority is $CD4^+$, only a small subset of human NKT cells expresses the CD8α chain. They are very abundant in mice and represent around 0.5% of the T cell population in blood and lymph nodes, 2.5% in spleen and approximately 30% in the liver. However, the frequency in humans is around 10% reduced in all organs, but can differ up to 100 times between different individuals. NKT cell are sub-classed into Type I and II NKT cells. Type I NKT cells (iNKT) are defined to

recognize α-galactosylceramide (αGC), whereas type II NKT cells recognize sulfatide presented by CD1d. Type II NKT cells express a more diverse set of TCRα chains compared to type I NKT cells.

The first and best described glycolipid found to stimulate NKT cells was αGC, isolated from a marine sponge [105]. αGC shows a strong affinity for CD1d in mice and humans. NKT cells produce a wide range of cytokines upon αGC stimulation. Already 1-2 hours after stimulation they produce Th1 cytokines such as TNF and IFNγ and Th2 cytokines such as IL-4 and IL-13. The regulation of Th1/Th2 cytokine expression is only partly understood. In mice, the rapid cytokine production is due to the presence of pre-formed mRNA stored in the cell, which enables a rapid response upon activation [106]. Injection of soluble αGC leads to a rapid production of IL-4, which is followed by a long lasting production of IFNγ and upregulation of CD40L on NKT cells. The majority of research has been focusing on IFNγ and IL-4 secretion, however, the picture is much broader and NKT cells have also been described to produce GM-CSF, TNF, IL-5, IL-10, IL-13, IL-17 as well as IL-21 [107, 108]. CD40-CD40L crosslinking results in upregulation of CD80/CD86 on DCs and IL-12 production, which in turn is critical to enhance the activation of NKT cells and their IFNγ expression [109]. Interestingly, NK cell mediated killing was also enhanced shortly after αGC engagement due to IFNγ release by NKT cells [110]. However, activation of NKT cells through αGC leads to downregulation of their TCR, to massive apoptosis approximately 3 to 4 days after exposure and to long lasting unresponsiveness [111, 112]. Interestingly, injection of αGC-loaded DCs induced a prolonged IFNγ response and was more potent in reducing metastasis in a B16 melanoma model compared to soluble αGC alone [113]. This suggests that αGC bound to DCs is more potent than soluble αGC in tumour therapy.

Apart from recognizing αGC, NKT cells are important in inducing immunity against bacteria like *Sphingomonas*. They recognize microbial α-glycuronylceramides, which can be found in gram-negative and lipopolysaccharide negative bacteria [114]. In mice and humans different self-ligands have also been described, however, the physiological role for these ligands remains unclear. Isoglobotrihexosylceramide (iGb3), a glycosphingolipid, was described to bind CD1d and activate NKT cells [115]. However, later on, its importance was questioned by showing that mice lacking iGB3 synthase develop a normal population of NKT cells [116]. In 2011 the endogenous ligand β-D-glucopyranosylceramide (β-GlcCer) was described to accumulate after TLR stimulation and to translate an innate TLR signal into an activation signal for iNKT cells [117]. NKT cells can function as an enhancer for the immune response and activate different immune cells by expression of different cytokines. This makes them a well suited target for cancer immunotherapy.

1.3.2 Anti-tumour Function of iNKT Cells

NKT cells play an important role in anti-tumour immunity as well as in immune surveillance, and mice lacking NKT cells are more susceptible to MCA-induced sarcomas and B16 melanomas [8, 118]. Interestingly, this effect was dependent on IFNγ expression by NKT

cells and independent of perforin. The anti-tumour effect mediated by αGC is highly dependent on IFNγ production by NK and NKT cells [119], which leads to activation of DCs and to subsequent IL-12 production. This can augment the adaptive immune response by activating cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells [120, 121]. NKT cells do not only mediate anti-tumour effects through activation of other immune cells, they can also directly target and kill CD1d-bearing tumour cells through similar mechanisms as used by NK cells and $CD8^+$ T cells such as perforin [122], TRAIL [123] or Fas ligand [124]. In addition, human tumours have been shown to express specifically glycosylated gangliosides [125], which are natural ligands for CD1d and can be presented on B cells to activate NKT cells [126]. Many tumour cells downregulate the expression of CD1d and are invisible for NKT cell mediated killing, which can lead to enhanced metastasis due to reduced control mechanisms in the primary tumour [127]. However, IFNγ dependent activation of other immune cells is still functional. Interestingly, type I and II NKT cells can have different functions in the tumour, while NKT I cells can have a protective role, NKT II cells can be immunosuppressive and secrete anti-inflammatory cytokines [128].

1.3.3 Immune Regulatory Type II NKT Cells in Cancer

There is also evidence that NKT cells can negatively influence anti-tumour immune responses. In certain tumour models NKT cells produce IL-13, which in turn activates myeloid ($GR1^+$ $CD11b^+$) cells to produce TGFβ that inhibits CTL function [129, 130]. In addition, CT26 colon carcinoma metastasis in the lung was greatly reduced in $CD1d^{-/-}$ mice. The effect was dependent on $CD8^+$ but not on $CD4^+$ T cells [131]. In summary, certain subsets of NKT cells can induce immunoinhibitory effects by producing IL-13. Further studies showed that type II NKT cells are sufficient for downregulating immune surveillance [132] and that injection of sulfatide, a type II NKT cell ligand, leads to higher metastatic burden in a CT26 colon carcinoma model [133]. Therefore, the understanding of NKT cell subsets and their activating and regulatory ligands is of major importance for developing new cancer therapies.

1.3.4 NKT cells in Cancer Immunotherapy

Promising results in many tumour models in mice led to several clinical trials using αGC in cancer patients. Intravenous injection of αGC in cancer patients did not induce significant biological effects. Only in patients with high NKT cell frequencies the production of cytokines was detected [134]. Injecting αGC-loaded DC led to better NKT cell activation and improved tumour control in mice [113]. However, several phase I clinical trials with this approach showed wide variation due to differences in NKT cell frequencies in the patients. Co-administration of NKT cells and αGC-pulsed DC induced significant anti-tumour immunity [135]. Furthermore, many cancer patients have impaired NKT cells, they fail to proliferate *ex vivo* [136] or are skewed towards a Th2 cytokine production [137], which challenges NKT cell-based therapies.

1.4 EXTRACELLULAR VESICLES

1.4.1 Exosomes

1.4.1.1 General overview

All cells investigated until today release small vesicles, which have important physiological functions and can be found in body fluids like plasma, urine, breast milk or saliva [138-140]. These vesicles differ in size, cellular origin and molecular composition. Extracellular vesicles carry important cargo and can transfer proteins, lipids and RNA to other cells [141, 142]. Intercellular communication is an important mechanism in all organisms and can be mediated by either small molecules such as hormones, growth factors or cytokines and act directly on the releasing cell (autocrine) or on other cells (endocrine). Direct cell to cell contact in proximity is mediated by desmosomes, tight junctions and gap junctions. A mechanism which has been overlooked is the intercellular communication via extracellular vesicles like exosomes and microvesicles.

Extracellular vesicles elicit important biological functions and act as messengers in immune activation and regulation in different malignancies. On one hand they can drive inflammation and act as immune activators; on the other hand tumour-derived vesicles downregulate the immune system through immune-suppressive molecules and can transfer pro-metastatic proteins to distant sites and facilitate metastasis [142, 143]. Thus, extracellular vesicles became a focus of interest for their use as therapeutic agents, as biomarkers or as a target for future cancer therapies.

The term exosomes was first used in 1981 when the group of Trams showed that extracellular vesicles are associated with adenosine production [144]. The first breakthrough paper was published by Johnstone and colleagues in 1987 where they extensively described the characteristics of exosomes. They showed that the transferrin receptor is lost during reticulocyte to erythrocyte maturation and is released via exosomes into the extracellular space [145]. In 1996, it was shown that B lymphocyte-derived vesicles carry MHC molecules and were able to stimulate cognate T lymphocytes [146]. This finding raised the interest for using exosomes in cancer therapy. Later on, the discovery of tumour-derived exosomes opened up a new field of research and scientists were interested in their role in cancer metastasis and tumour microenvironment and their use as biomarkers.

Extracellular vesicles are a heterogeneous group and differ in size, lipid and protein composition. Today three main subgroups of extracellular vesicles have been described: **i)** apoptotic bodies, **ii)** microvesicles/ectosomes and **iii)** exosomes. Apoptotic bodies are released during cell death during which cytosol and organelles are packed into the blebbing plasma membrane [147]. They have a wide size range and may contaminate other vesicle pellets. Microvesicles are released directly from the surface of the cell while exosomes have endosomal origin. Microvesicles and exosomes can be clearly distinct from apoptotic bodies by their proteomic profiles [148].

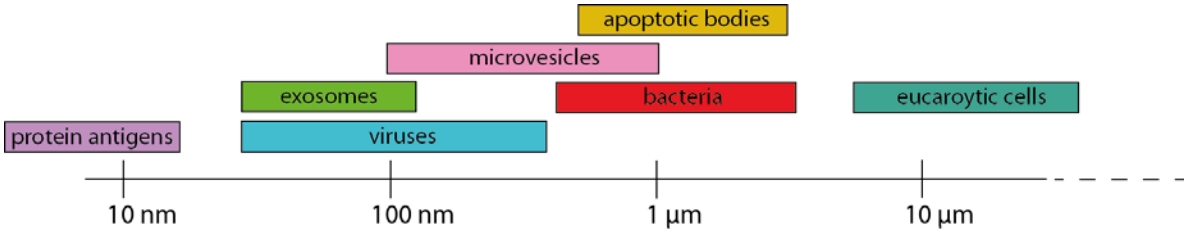

Figure 2: Size distribution of extracellular vesicles in comparison to bacteria, viruses and eukaryotic cells

1.4.1.2 Definition

Exosomes are extracellular vesicles with endosomal origin with an approximate diameter of 30 to 150 nm and are released by all kind of cells investigated until this day. Due to their endosomal origin they do not express mitochondrial or endoplasmatic reticulum (ER)-derived proteins. They carry proteins, lipids and nucleic acids and can transfer information from one cell to the other. Exosome are usually isolated using different centrifugation and filtration steps with a final ultracentrifugation step at 100´000 x g. Microvesicles on the other hand pellet at 10´000 x g. More recently, exosomes and microvesicles are also isolated by size exclusion chromatography. Visualization by transmission electron microscopy (TEM) reveals exosomes with a cup-shaped morphology, which might be due to fixation and embedding methods. Exosomes without any fixation show a round morphology. Flotation on a sucrose gradient defined them at a density level of 1.13 to 1.19 g/ml. Currently, there is still no specific marker for exosomes. Therefore, vesicles are best characterized by different methods such as western blot, electron microscopy, FACS and nanoparticle tracking analysis. Selection of subpopulations of exosomes can be achieved by different methods such as immune-affinity purification [149, 150], isoelectric gradients [151], size based methods [152] and the classical sucrose gradient. Interestingly, subpopulations isolated by sucrose gradients have distinct proteomic and genomic profiles and are involved in different pathways [153]. However, subpopulation can vary depending on the isolation technique and cell of origin. In general, proteins enriched in exosomes and considered as exosomal markers include tetraspanins (CD9, CD63 and CD81), MHC class I and II molecules, heat shock proteins (HSP70, HSP90) [154, 155] and proteins from the endosomal sorting complex required for transport (ESCRT) components such as TSG101 and Alix [156] (Figure 3). Furthermore, exosomes can also contain cytoskeletal proteins such as actin and tubulin [157]. However, tetraspanins have also been shown to be associated with apoptotic bodies and microvesicles [158] and only a subpopulation of exosomes may contain CD63 and Tsg101 [159, 160]. In addition, several proteins used as exosomal markers can also be expressed in larger vesicles pelleting at lower speeds, this includes flotillin, heat shock proteins or MHC class I and II molecules. Lately, syntenin-1, Tsg101, ADAM10, EHD4 and Annexin XI have been described to be a better marker for exosomes compared to CD9 and CD63 [160].

Exosomes express a specific set of glycoproteins, which is different from the glycol pattern of the mother cell [161] and interestingly also from apoptotic bodies [162]. The glycosylation pattern of exosomes can vary depending on physiological conditions and might have an important function in targeting other cells or in systemic biodistribution [162, 163]. Beside a specific glycol-pattern, exosomes are enriched in certain lipids such as sphingomyelin, cholesterol and phosphatidylserine compared to the parent cell [164]. They can also express ceramide [165], flotillin and phosphatidylethanolamine, which leads to a more rigid membrane and contributes to their stability in the extracellular environment [166].

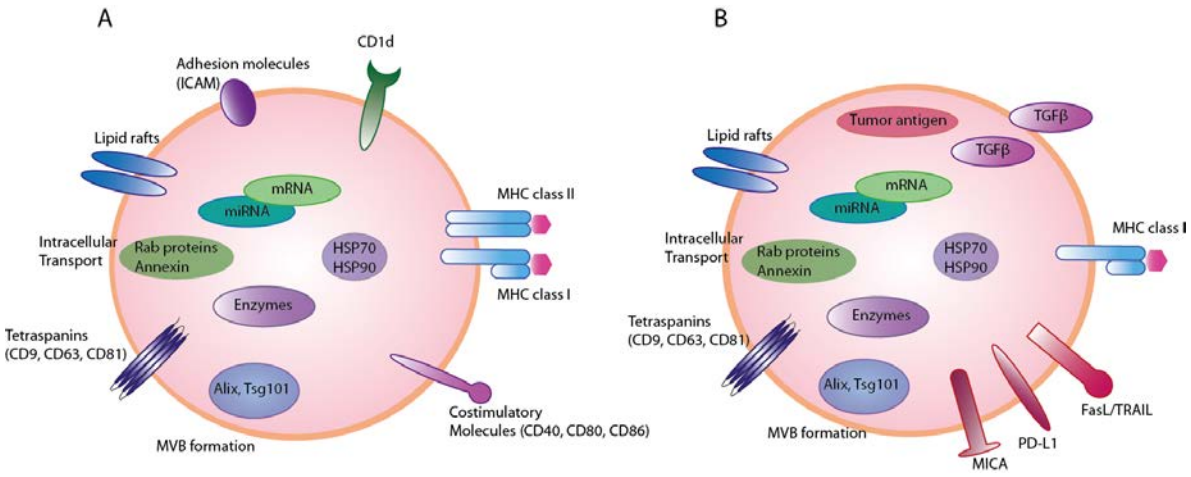

Figure 3: Protein components of exosomes **A)** DC-derived exosomes express costimulatory and adhesion molecules and CD1d, B) Tumour-derived exosomes express immunoinhibitory molecules, oncoproteins and enzymes promoting tumour progression.

Exosomes contain intact RNA with a size range of up to 700 nucleotides (nt), while cellular RNA can be 400 to 12 000 nt long [167]. They may contain mRNA and mRNA fragments [141], miRNA [168] and short DNA sequences [169]. Several studies report absence of 18S and 28S ribosomal RNA (rRNA) [158, 170], however, depending on the isolation protocol certain publications describe the presence of rRNA in exosomes [153, 171]. Furthermore, microvesicles (here used as another name for exosomes) were described to contain mainly ssDNA [172]. Variability between studies exists and depending on methodology, isolation protocol and purity of the isolated vesicles results might differ. Interestingly, the RNA content of exosomes does not resemble the RNA content of the parent cell. Some RNA species are enriched in exosomes [141, 142, 170] and a specific sequence motif has been described, which induces loading of the microRNA into exosomes [173]. This indicates a selective and active process for RNA loading into exosomes. Heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2b1) binds specifically the sequence motives and sorts miRNA into exosomes, mutagenesis of these motifs changes the loading of miRNA into exosomes [173]. miRNA content can differ between MV and exosomes and in different exosomal subpopulations [174]. Remarkably, exosomes can transfer RNA between different

cells *in vitro* with translation of the protein in the recipient cells [141, 142]. Therefore, exosomes might function as communication vesicles and transfer RNA, which is protected in the exosome lumen from degradation by RNase. In contrast, RNA can also be associated with RNA-binding proteins (Argonaut-2, Ago2) [175] or lipoproteins [176] to prevent their degradation. Certain mRNA species were exclusively associated with vesicles or protein complexes but the majority of RNA was associated with RNA-binding proteins and not vesicles [175]. However, it is still not clear how cells take up miRNA associated to Ago2. The association to protein complexes, lipoproteins or vesicles might greatly affect the biological function and uptake by other cells.

1.4.1.3 Exosome Biogenesis

During maturation of an early to a late endosome, intra luminal vesicles (ILV) are budding off from the membrane into the endosome lumen leading to the formation of multivesicular bodies (MVB). MVB either fuse with the lysosome, where all the content is degraded by hydrolases, or with the plasma membrane (Figure 4). The lipid content of an MVB fusing with a lysosome is significantly different to an MVB fusing with the plasma membrane [177] and cholesterol-poor MVB are normally degraded in lysosomes [178]. Once the MVB has fused with the plasma membrane, the ILVs are released into the extracellular space and are now called exosomes. There are several different mechanisms for the formation of MVB, the best described and investigated is dependent on the endosomal sorting complex required for transport (ESCRT). Apart from the ESCRT machinery, tetraspanins and lipids also play major roles in exosomes biogenesis.

The ESCRT complex consists of four different subunits, ESCRT-0, -I, -II, and –III. ESCRT-0 is important in binding mono-ubiquitinated cargo proteins and binds via Hrs to TSG101 of ESCRT-I. Silencing of Hrs in mammalian cells leads to a reduced formation of ILVs [179]. ESCRT-I and II are responsible for the membrane deformation process and for the shipment of the cargo into the vesicles. ESCRT-I recruits ESCRT-II and finally ESCRT-II binds ESCRT-III. The dissociation of the vesicle is mediated by AAA ATPase Vps4. A study silencing multiple components of the ESCRT complexes showed epidermal-growth factor (EGF)-independent formation of MVB [180]. Thus, there must be other molecules involved in ILV budding. Recently, it has been shown that depletion of Hrs induces a CD63 dependent formation of ILVs in HeLa cells [181]. Cells deficient of four subunits of the ESCRT machinery can still produce CD63 positive MVB. Apart from CD63 and ESCRT dependent formation of ILV, lipid induced budding has been described as well. The sphingolipid ceramide has been shown to be important for ILV formation in mouse oligodendroglia cells [165]. Blocking of neutral sphingomyelinases enzyme (snMase) (which is important for ceramide synthesis) reduced exosome formation. In contrast, the impairment of snMase does not inhibit the formation of MVB in human melanocytes. Different cell lines can use different mechanisms for exosome formation. Furthermore, other lipids like cholesterol and lysobisphosphatidic acid (LBPA) and phosphatidic acid have been suggested to be involved in exosome biogenesis [182-184].

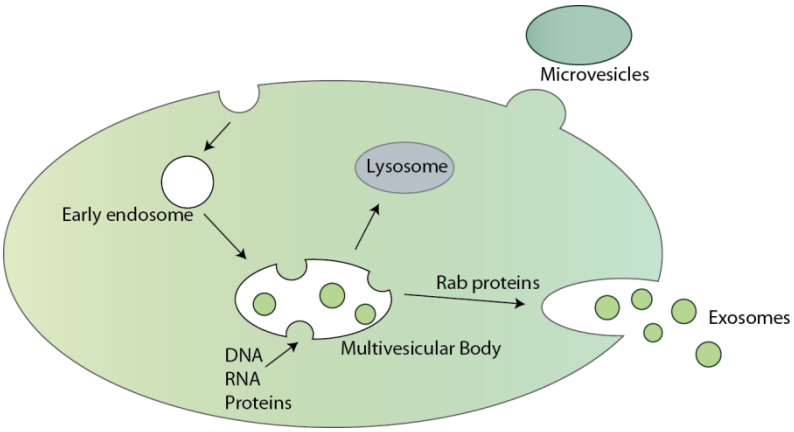

Figure 4: Exosomes are formed as intraluminal vesicles (ILV) in the late endosomes resulting in multivesicular bodies. Upon fusion with the cell membrane, ILVs are released into the extracellular space, and are then called exosomes. In contrast, microvesicles bud off directly from the cell plasma membrane.

1.4.1.4 Exosome secretion

Rab GTPases are important proteins involved in membrane trafficking, vesicle transport and membrane fusion. Many studies have investigated the involvement of Rab proteins in exosome secretion. Already in 2002 it was shown that secretion of exosomes by erythroleukemia cell line K562 is modulated by Rab11 [185]. In 2010 Rab27 and Rab35 were identified to play an important role in exosome secretion. Inhibition of Rab35 in a murine oligodendroglia cell line led to accumulation of ILVs and to a reduced exosome secretion [186]. Ostrovski and colleagues found that Rab27 isoforms effect exosome secretion in HeLa cells and shRNA induced silencing of Rab27a/b led to a reduced exosome release [187]. However, Rab27a silencing in 4T1 cells leads to reduced expression of CD63, Tsg101, Alix and Hsc70 on released vesicles but not of CD9 and MFGE8. Interestingly, CD9 and MFGE8 can also be found on microvesicles, pelleted at 10´000 x g. The authors propose that the 100´000 x g exosome pellet is a mixture of vesicles with different cellular origin [188]. Several Rab proteins have been shown to be important in cancer progression and metastasis. For example, gastric cancer patients have a lower survival rate when expressing high levels of Rab40b. [189] Recently, two studies correlated expression levels of different Rab proteins in cancer tissue with clinical status of these patients. In pancreatic cancer patients the expression level of Rab27a in the cancerous tissue is associated with tumour stage and vascular invasion [190]. The upregulated expression of Rab proteins in cancer patients might induce an enhanced exosome secretion from the cancer cells, which in turn promotes cancer invasion and progression.

1.4.2 Microvesicles

Microvesicles (MV) were first described in shedding from activated erythrocytes and platelets [191] and were shown to be important in coagulation [192]. MVs are shed directly from the plasma membrane after a process of protein and lipid rearrangement. Like exosomes, MVs are shed from a variety of cell types and can be found in body fluids like urine, plasma and ascites [193-195].

Changes in the activation status of a cell and subsequent calcium accumulation in the cytosol lead to rearrangement of the lipid membrane and to exposure of phosphatidylserine to the external lipid layer. The asymmetry of the cell membrane and disruption of the cytoskeleton induces membrane budding [193]. There are no specific proteins which have been described to be a marker for MVs. Certain proteins were described to be part of the budding process. ARF6, a GTP binding protein, has been shown to be involved in membrane budding and MV release in tumour cells [196]. In addition, certain studies show that MV budding can be mediated by Tsg101 and Vps4 (part of the ESCRT machinery), which have previously been described to mediate exosome biogenesis [197]. Interestingly, proteins from the ESCRT-0 complex are often not involved in plasma membrane budding [198], therefore the presence of ESCRT-0 supports the MVB origin of the vesicle. As already discussed for exosomes, ceramide and tetraspanins are also important for the MV budding process. Ceramide also accumulates in the membrane of MV and acid sphingomyelinase is important for the budding process [199]. In addition, tetraspanins can form special microdomains in the plasma membrane, which enables MV budding [158]. MV have been shown to express an overlapping but distinct different protein profile compared to exosomes [153] (Table 1).

Table 1: Overview on exosomes and microvesicles

	Exosomes	**Microvesicles**
Size	30 – 150 nm	100 – 1000 nm
Other name	TEX, DEX, tolerosomes, prostasomes	ectosomes, microparticles
Molecular composition/Markers	Flotillin, Alix, Tsg101, tetraspanins (CD9, CD63, CD81)	Tissue factor, flotillin, parts of the ESCRT complex
Sedimentation	100´000 x g	10´000 – 20´000 x g
Lipid composition	cholesterol sphingomyelins, phosphatidylcholine, phosphatidylethanolamine [166] ceramide	Similar to plasma membrane, aminophospholipids, phosphatidyl-serine and -ethanolamine, in outer leaflet [200]
Nucleic acids	mRNA, miRNA, small RNA	rRNA [158]
Origin	Late endosome, MVB	Plasma membrane
Assembly/budding process	ESCRT, CD63, ceramide dependent [165]	TSG101, ARRDC1- mediated [201]

1.5 EXOSOMES IN THE IMMUNE SYSTEM

1.5.1 Exosomes in Immune Activation

The first time exosomes drew interest in the immunological society was in 1996 when antigen-presenting cells were described to secrete vesicles expressing MHC class II molecules. Raposo and colleagues showed that exosomes from Epstein-Barr Virus transformed B cells express MHC/peptide complexes, costimulatory and adhesion molecules and can directly stimulate proliferation of a cognate $CD4^+$ T cell line [146]. This was the first evidence that exosomes from immune cells might play an important role in cell to cell communication in the immune system and that extracellular vesicles are sufficient to activate T cells *in vitro*. At the same time different research groups became interested in DCs loaded with acid eluted tumour peptides as cancer therapy. Exosomes from mature bone-marrow derived dendritic cells (BMDC) pulsed with acid eluted tumour peptides primed tumour-specific $CD8^+$ T cells in a mouse tumour model, which led to eradication of established tumours. Furthermore, the induction of a tumour-specific CTL response was fully dependent on syngeneic MHC/peptide complex on exosomes and no effect was seen after injection of allogeneic exosomes [202]. However, in another system exosomes from BMDC indirectly loaded with the major $CD8^+$ T cell peptide SIINFEKL were unable to induce antigen-specific T cells *in vivo*. Interestingly, only whole OVA antigen-loaded exosomes induced antigen-specific $CD8^+$ T cells. This effect was dependent on the presence of $CD4^+$ T cells and partially on marginal zone B cells [203]. These results were supported by a recently published study where peptide-loaded B cell-derived exosomes were weak in inducing antigen-specific $CD8^+$ T cell responses [163].

Mature DC-derived exosomes express high levels of MHC class I and II molecules, CD54 (ICAM-1) and the costimulatory molecules CD80 and CD86. Hence, they can possibly activate $CD4^+$ and $CD8^+$ T cell responses. Exosomes form mature DC are much more efficient in activating and inducing T cell responses compared to exosomes from immature DCs, which express lower levels of MHC and costimulatory molecules [204, 205]. DC-derived exosomes can activate T cell clones, cell lines and primed $CD4^+$ and $CD8^+$ T cells directly [139, 146, 206]. However, certain studies showed that the exosome-induced T cell activation was minor and a strong T cell response could only be induced in the presence of DCs [207, 208]. This might be an explanation for the poor ability of DC-derived exosomes to stimulate naïve T cells, where the threshold for activation is much higher compared to T cell clones and memory T cells [209]. Furthermore, the small size of exosomes and the low amounts of MHC/peptide complexes on exosomes can also be a limiting effect for their stimulatory capacity. Hsu and colleagues showed that exosomes, which are directly loaded with peptide in mildly acid conditions, were more effective in inducing T cell proliferation compared to indirectly loaded exosomes *in vitro*. This augments the numbers of MHC molecules loaded with peptides significantly [210]. In conclusion, for the activation of naïve T cells the uptake and processing by DC is likely necessary [209].

1.5.1.1 Exosome binding and uptake

The immunogenicity and uptake of exosomes likely depends on certain molecules expressed on the exosome surface. Expression of ICAM-1 increases exosomal immunogenicity *in vitro* [205]. Vesicles released by *Drosophila* cells engineered to express MHC/peptide complexes and ICAM-1 shown to bind to cognate T cells efficiently and activated them in the presence of CD80/CD86 [211]. The presence of ICAM-1 on exosomes might influence the binding of exosomes to T cells and is crucial for T cell stimulation. Mature DC-derived exosomes contain high levels of ICAM-1 and can efficiently bind to LFA-1 expressed at high levels by on T cells, macrophages, B cells and $CD8^+$ DCs. T cells can be the target of MHC class II expressing DC-derived exosomes [212] Interestingly, only activated T cells (through CD3/CD28) can recruit DC-derived exosomes via ICAM-1/LFA-1 interaction. DC-derived exosomes can bind to T cells during a cognate DC-T cell interaction [206]. Furthermore, DC-derived exosomes can also interact directly with DC through expression of ICAM-1 on exosomes and LFA-1 on DC. LFA-$1^{-/-}$ DCs do not interact with exosomes and cannot induce T cell responses [205, 213].

The interaction of exosomes with immune cells in lymphoid organs like spleen and lymph nodes is crucial for the induction of an exosomal induced immune response. Capturing of intravenously injected exosomes in the spleen is mediated by $MOMA^+$ macrophages, ER-TR9 macrophages and $CD11c^+$ DCs of the marginal zone. They can transfer exosomal proteins directly to the follicle [87]. Similarly, already 5 min after injection Siglec-1 (CD169) expressing macrophages in the marginal zone and in the subcapsular sinus of lymph nodes capture B cell-derived exosomes, which are enriched in α-2,3-linked sialic acid. Interestingly, depletion of CD169 led to binding to $SIGNR1^+$ macrophages and $F4/80^+$ red pulp macrophages [163]. Just recently, $CD169^+$ macrophages were shown to play an important role in clearing tumour-derived extracellular vesicles and blocking their dissemination and metastasis [214]. Other studies describe $CD8^+$ DCs to be important for exosome uptake compared to $CD8^-$ DCs and plasmacytoid DCs. Already 24 hours after i.v. injection BMDC-derived exosomes were found in the T cell zone in the spleen and on follicular DCs [215, 216].

Exosomes also express milk-fat globule E8 (MFG-E8; known as lactadherin) and phosphatidylserine (PS). Both can act as opsonins and enhance phagocytosis and uptake. PS is normally only expressed on the outer plasma membrane leaflet during apoptosis. MFG-E8 can interact directly with PS but also contains an integrin binding domain for cell interaction. Blocking of PS and RGD sequences (e.g. MFG-E8) reduces uptake of exosomes by DCs [87]. Furthermore, exosomes have been shown to bind the C-type lectin receptor DEC205 on DCs [217], which is highly expressed on marginal zone macrophages to capture blood-borne antigens. Antigen targeting to DEC205 leads to enhanced uptake and cross-presentation and to more efficient priming of the immune system [218]. Thus, binding of exosomes to DEC205 on DCs might induce cross-presentation and activation of $CD8^+$ T cells.

Uptake of exosomes by DCs is an active process and can be reduced by adding cytochalasin D which interrupts actin polymerization or by incubation at 4 °C. In addition, blocking of tetraspanins CD9 and CD81 also reduced uptake of exosomes by DCs [87]. Once exosomes are taken up by DCs, the MHC/peptide complex can be degraded or reused on the DC surface for T cell simulation. The transfer of exosomal MHC/peptide complexes to MHC class I/MHC class II deficient mature DC have been shown to induce T cell proliferation by recycling the exosomal MHC/peptide complex [208, 209]. Furthermore, exosomes can transfer HLA-A2/MART1 peptide complex to APC lacking the HLA-A2 molecule [210]. In conclusion, DCs and macrophages play a crucial role in initiating exosome induced immune responses in lymphoid organs and capture exosomes through specific surface receptors.

1.5.2 Immunomodulatory Exosomes in Cancer Therapy

As described above, exosomes can potently stimulate the immune system. Here follows an overview of reports where either tumour-derived or DC-derived exosomes have been used in preclinical cancer models (Table 2) and clinical trials (Table 3). In some cases, DC-derived exosomes were loaded with tumour peptides or whole antigens to elicit a specific anti-tumour response [202, 219]. Several studies potentiated the immune response by adding innate stimuli [220-222]. On the other hand tumour-derived exosomes were used as antigen source in several vaccination models [223-225] and in some cases the immunogenicity of TEX was enhanced by different methods [154, 226-229]. In summary, *in vivo* effects are well described in preclinical models; unfortunately, the efficiency is very low in the clinical trials.

Table 2: Summary of results of exosome-based therapies in preclinical models

Cancer type	Exosome vaccine	Injection route	Outcome	Ref.
Mastocytoma, mammary carcinoma	Mature BMDC-derived exosomes, loaded with acid eluted cancer peptides *therapeutic model*	i.d. 3-5 µg	T cell dependent eradication of established tumours	[202]
Plasmacytoma J558	Tumour cell derived exosomes *vaccine model*	s.c. 2,5-5 µg	T cell dependent immunity against plasmacytoma	[223]
Colon carcinoma CT26	MUC1 transfected cell lines, CT26 murine colon carcinoma and TA3HA mammary carcinoma *vaccine model*	i.d. 4 or 20 µg, with Incomplete Freund´s Adjuvant	MUC1 carrying exosomes from CT26 and TA3HA induced anti-tumour effects	[224]
SW480 human colon adeno-carcinoma in nude mice	exosomes from heat-stressed (HS) carcino-embryonic antigen (CEA) positive tumour cells *therapeutic model*	s.c. 5 µg	higher CTL induction after injection of exosomes from HS tumour cell, better survival after adoptive transfer of splenocytes of HS-exosome injected mice in a SW480 tumour model	[226]
B16F10 melanoma co-expressing HLA-A2/gp100	BMDC-derived exosomes, MelanA/Mart1/gp100 peptides and CpG/ODN in combination with cyclophosphamide (CTX) *treatment model*	10 µg foodpad	enhanced CTL response and survival in a B16F10 tumour model	[220]
L1210 murine B lymphocytic leukemia	Tumour cell line-derived exosomes *vaccine model*	s.c. 2.5 or 5 µg	Prolonged survival of the exosome vaccinated groups	[225]
BL6-10$_{OVA}$ tumour	BMD- derived exosomes loaded with OVA or EG7 derived exosomes *vaccination model*	i.v. 10 µg	BMDC exosomes induced more antigen-specific $CD8^+$ T cells and less metastasis compared to tumour derived exosome	[230]
EG7 lymphoma	EG7-derived exosomes with bound staphylococcal enterotoxin A (SEA) *vaccination model*	s.c. 10 µg	Induction of more antigen-specific T cells and longer survival after injection of EG7-SEA exosomes	[227]
L1210 murine B lymphocytic leukemia	BMDC-derived exosomes, matured with L1210 antigen and LPS, in combination with polyI:C and CTX *therapeutic model*	s.c. 10 µg	Combination therapy with polyI:C, CTX and exosomes leads to prolonged survival compared to the treatment alone	[221]

MUC1 expressing CT26 colon carcinoma or B16 melanoma	HSP70 enriched tumour exosomes, heat induced *therapeutic model*	i.d. 20 µg 3x every 2nd week.	HSP70 enriched exosomes prolonged survival even in allogeinic mice	[154]
J558 myeloma	myeloma cell line J558 expressing tumour antigen P1A, engineered to secret either IL-2, TNFα or IFNγ *vaccination model*	i.v. 30 µg	Exosomes from tumour cells expressing TNFα showed the best anti-tumour immunity	[228]
3LL tumour, lung carcinoma	Heat stressed tumour cell-derived exosomes from 3LL lung cancer *therapeutic model*	i.t. 5 µg	Heat stressed tumour-derived exosomes attract DC and activate T cells more potently, reduce tumour growth	[231]
B16F1 melanoma	MHCII expressing B16F1 melanoma-derived exosomes (CIITA transduced) *therapeutic model*	i.d. 5-20 µg	Induce more activation of immune cells, Th1 skewing	[232]
Xenograft breast cancer cells (HCC70) in RAG2$^{-/-}$ mice	HEK293 expressing GE11 which binds to overexpressed EGFR on tumour cells and transfected with let-7a miRNA *therapeutic model*	i.v. 1 µg, 1 x/week for 4 weeks	GE11 positive exosomes bind to tumour and reduce tumour growth via let-7a, let-7 expression in cancer cell lines alters cell cycle progression	[233]
Glioma xenograft model 9L	MSC transfected with miR-146b plasmids, (mir146b has been shown to reduce cell motility) *therapeutic model*	i.t. 50 µg	One i.t. injection of exosomes carrying mir-146b led to reduced tumour growth in rats	[229]
B16 melanoma	BMDC-derived exosomes loaded with OVA and αGC *therapeutic model*	i.v. 40 µg, 1 or 2 times	Exosomes loaded with OVA and αGC prolonged survival, no induction of anergic NKT cells	[222]
A549 non-small cell lung cancer	Rab27a overexpression in the human non-small-cell lung cancer cell line A549 *vaccination and therapeutic model*	s.c. 10 µg 4x before/after tumour injection	Exosomes from Rab27a overexpressing cell line induce better DC maturation, more Th1 cytokines and a stronger anti-tumour immunity	[234]
3LL tumour, lung carcinoma	3LL lung tumour carcinoma cells transfected with CD40L *vaccination and therapeutic model*	s.c. 10 µg	exosomes activate DC more efficiently and induce more CTL leading to more efficient anti-tumour immunity	[235]
human breast cancer cell line MDA-MB-231	immature mouse DC cell line deliver doxorubicin (Dox), engineered to target integrins *therapeutic model*	i.v.3mg/kg	Exosomes containing Dox and a targeting domain induce slower tumour growth	[236]

Table 3: Summary of results of exosome-based therapies in clinical trials

Cancer type	Exosome vaccine	Injection route	Clinical outcome	Ref.
Non-small cell lung cancer patients	DC-derived exosomes loaded with MAGE tumour peptides *therapeutic model*	s.c. (90%), i.d. 10% 0.13×10^{14} MHCII molecules	No toxicity, poor MAGE-specific T cell response	[219]
Metastatic melanoma patients	DC-derived exosomes loaded with MAGE tumour peptides *therapeutic model*	s.c. (90%), i.d. 10%, 0.13 to 0.4 $\times10^{14}$ MHCII molecules	No toxicity, stable disease in one patient, no MAGE specific CTLs in the blood	[237]
Colorectal cancer patients	Ascites-derived exosomes (Aex) in combination with GM-CSF *therapeutic model*	s.c. 100, 200, 300 or 500 µg Aex and 50 µg GM-CSF	Aex and GMCSF together induce cytotoxic T lymphocytes	[238]
Non-small cell lung cancer patients	IFNγ matured monocyte-derived DC *therapeutic model*	i.d.	Boost of NK cell activity, no tumour-specific T cell induction	[239]

1.5.3 Exosomes in Immune Suppression

Tolerosomes. In 2001 it was described for the first time that OVA fed mice express MHC class II positive exosome like-vesicles in the plasma, which are able to induce tolerance and suppress OVA-specific immune responses in syngeneic mice [240, 241]. A similar study showed that plasma-derived exosomes from KHL (keyhole limpet hemocyanin protein) immunized mice are immunosuppressive in a delayed-type hypersensitivity model. This effect was dependent on antigen, MHC class II expression and FasL on intact exosomes. Interestingly, mechanical disruption of exosomes resulted in loss of the immunosuppressive activity [242]. It has been discussed that commonly encountered antigens induce immunosuppressive effects to reduce the risk of developing chronic inflammation and autoimmunity. Furthermore, exosomes isolated from bronchoalveolar lavage fluid of mice intranasally exposed to an allergen, induced lower IgE levels and Th2 cytokine levels in a mouse model of allergic airway inflammation [243].

Mesenchymal stem cell-derived vesicles. Mesenchymal stem cells (MSC) have been reported to induce immunosuppressive effects and were used in phase I and II clinical trials in type I diabetes, Crohn´s disease and cardiovascular disease [244]. Interestingly, several studies describe that injection of conditioned MSC media suppressed inflammation as efficient as the MSC alone [245]. Later on, the effect was described to be exosome dependent. MSC-derived exosomes reduced infarct size in a mouse model of myocardial ischemia by reducing oxidative stress and promoting cell survival factors [246, 247].

Exosomes in breast milk and pregnancy. Exosomes can be found in breast milk [248] and have been shown to induce regulatory T cells *in vitro* [139] and to carry immunomodulatory miRNA [249]. Pregnancy has been shown to alleviate different chronic inflammatory diseases like rheumatoid arthritis [250]. It is possible that placenta-derived exosomes can be part of the modification of the immune status of the mother. Expression of NKG2D ligand such as MICA/B on placenta-derived exosomes inhibit NKG2D expression on NK cell, $CD8^+$ T cells and γδ T cells leading to reduced cytotoxic activity [251, 252]. Furthermore, placenta-derived exosomes can alter gene expression in T cells [253] and inhibit T cell signalling via downregulation of CD3-zeta chain mainly on $CD8^+$ T cells [254]. In addition, it has been shown that placenta-derived exosomes express FasL and TRAIL, which can induce apoptosis in Jurkat cells to induce an immune privileged site in the uterus [255]. Taken together, these results suggest that placenta-derived exosomes are playing an important role in influencing the immune status of the mother.

Exosomes in transplantation. Immunosuppressive exosomes were used as treatment to reduce allograft rejection. Treatment with immature donor DC-derived exosomes in combination with a sup-optimal dose of rapamycin prolonged survival after heart transplantation significantly [256]. Similar results were obtained with treatment of DC-derived exosomes and a drug inhibiting DC maturation. The combination of exosomes and drug prolonged survival markedly compared to drug treatment alone [257].

Regulatory T cell-derived exosomes. $CD4^+$ $Foxp3^+$ T cells express exosomes with immunomodulatory capacity. Rab27a/b silencing in Tregs leads to reduced exosome secretion and interestingly to enhanced systemic inflammation [258]. In addition, Treg-derived exosomes inhibit $CD4^+$ T cell activation and IFNγ and IL-2 production *in vitro*. This effect was inhibited following addition of a CD73 inhibitor [259]. The expression of CD39 and CD73, enzymes involved in adenosine production, has been shown to be crucial for the immune suppressive function of Treg cells [260, 261]. Recently, certain microRNA in Treg-derived exosomes have been shown to play an important role in immune modulation. miR-155, Let-7d and Let-7b in Treg exosomes are important in reducing systemic inflammation [258]. Treg-derived exosomes were injected after kidney transplantation and led to prolonged survival and improved function of the transplant [262]. In conclusion, Tregs secrete exosomes, which may play an important role in systemic immune suppression and could be used as a therapeutic agent.

Engineering of immune suppressive exosomes. Immature DCs carry low levels of MHC and costimulatory molecules on the surface and can be immunosuppressive. DC genetically modified to express IL-4 or FasL can suppress collagen induced arthritis [263, 264]. Interestingly, also exosomes from bone marrow-derived DC treated with IL-10 or genetically modified to express FasL can suppress collagen induced arthritis [265, 266]. The mechanistic effect for how these vesicles suppress the immune response is still unclear. However, the effect was MHC class II and antigen dependent. MHC class II deficient exosomes did not suppress inflammation whereas MHC class I deficient vesicles were functional [266]. In

contrast, Yu *et al*. described that exosomes isolated from DCs expressing membrane-associated TGFβ inhibited progression of MOG-induced EAE in mice also in allogeneic mice [267].

1.6 TUMOUR-DERIVED EXOSOMES AND MICROVESICLES

Exosomes play an important part in communication between cancer cells. Recent results suggested that exosomes are involved in cancer growth, tissue remodelling and metastasis. Cancer cells release large quantities of extracellular vesicles, which can be found in the body fluid of cancer patients. Blood plasma from melanoma cancer patients is enriched in exosomes compared to healthy controls and exosome concentration in the serum correlates with prognosis [268]. In addition, metastatic cancer cells release exosomes expressing high levels of ESCRT-related proteins which indicates a high production rate of exosomes [269].

Metastasis. Invasion and metastasis is the main treatment failure and connected with poor prognosis in cancer. Metastasis involves acquiring of migratory capacity, losing epithelial phenotype, dissemination and establishment at distant sites [270]. Tumour-derived exosomes (TEX) have been shown to facilitate the migratory capacity and to induce metastasis and invasion. Melanoma-derived exosomes home to the sentinel lymph node and induce changes in the gene expression profile of genes involved in cell recruitment, extracellular matrix and vascular growth factor production, all of which facilitates metastasis to the draining lymph node [143]. Interestingly, miRNA released in exosomes from cancer cells can bind to TLR, which leads to tumour promoting inflammation that favours metastasis and tumour growth [271]. Exosomes from highly metastatic melanoma cells educate bone marrow cells and induce a greater tumour growth and lung metastasis. Furthermore, the exosome-associated oncoprotein MET induces enhanced bone marrow cell mobilization [268] and tumour-derived exosomes can directly promote specific organotrophic metastasis through organ specific expression of integrins [272]. Further understanding of the mechanism of how exosomes promote metastasis is needed to be able to therapeutically target the mechanism. Specific protein expression profile on TEX in the plasma of patients could be used as future biomarkers to distinguish patients with high metastatic burden from patients with low metastatic burden.

Angiogenesis. Different studies have investigated the influence of exosomes on promoting angiogenesis in the tumour microenvironment. One of the first studies investigating the influence of MVs on angiogenesis showed that platelet-derived MVs transferred the CD41 integrin to lung cancer cells and stimulated the MAPK pathway and led to an increase in the expression of specific matrix metalloproteases and cyclin D2 and upregulation of different angiogenic factors. [273]. Glioblastoma tumour cells release exosomes containing angiogenic factors and can stimulate endothelial cells to form tubules [142]. In addition, monocytes releasing exosomes containing miRNA-150 strongly induce endothelial tube formation and injection of anti-miR-150 into tumours in mice reduced formation of new blood vessels [274]. Human colon cancer-derived exosomes activate Egr-1, which leads to enhanced cell

migration of an endothelial cell line via the activation of ERK1/2 and JNK kinases [275]. Hence, exosomes influence the tube formation and the development of new blood vessels and thus contribute to tumour growth and invasiveness.

Transfer of oncogenic proteins. Tumour-derived exosomes can transfer certain information from the parent cell to other malignant or normal cells. TEX can carry oncoproteins from the parent tumour cell and transfer these to other cells [276-278]. An oncogenic form of the epidermal growth factor receptor (EGFRvIII) can be transferred to cells lacking this mutated receptor, which in turn leads to upregulation of anti-apoptotic genes and to anchorage independent growth [279]. Furthermore, exosomes from a colon cancer cell line expressing mutant KRAS are able to transfer mutated KRAS to a cell expressing wild-type KRAS which leads to an enhanced three-dimensional growth [280]. Transfer of oncoproteins by exosomes to other cells can be a mechanism to transform normal growing cells into cancer cells and leading to better tumour growth.

Breakdown of extracellular matrix (ECM). Exosomes and MVs can mediate metastasis by expressing enzymes important for extracellular matrix degradation to facilitate metastasis. EMPIRIN, a glycoprotein expressed by tumour cells, which induces expression of matrix metalloproteases in fibroblasts, has been shown to be shed in vesicles [281, 282]. Prostate cancer-derived exosomes can influence differentiation of mesenchymal stem cells to cells producing VEGF, HGF and metalloproteases [283].

Hypoxia. Hypoxia is a common hallmark of many tumours [284], it drives tumour formation and is associated with metastasis and poor prognosis [285]. Low oxygen levels in the tumour lead to changes in the expression of genes involved in angiogenesis, metastasis and invasion mediated by hypoxia inducible factor (HIF) [286]. Hypoxic breast cancer cells augment release of MVs by HIF-dependent upregulation of Rab22a. These hypoxia-induced MVs promote invasion and metastasis in a Rab22a dependent manner [287]. Several studies describe upregulation of exosome production under hypoxic conditions including expression of certain miRNA [288, 289] or IL-10/TGFβ [290, 291], which in turn leads to enhanced motility and endothelial tube formation and immunosuppression, respectively.

pH. Low pH levels can be detected in the tumour microenvironment and might influence activities of enzymes involved in ECM degradation. Tumour cells release more exosomes under acidic conditions than in buffered conditions and, interestingly, these exosomes fuse more efficiently with the plasma membrane of tumour cells [292]. This might be a mechanism to enhance the transfer of proteins or RNA via exosomes in the tumour.

1.6.1 Immune Suppressive Exosomes in Cancer

Already in 1985 Poutiaska and colleagues showed that vesicles released by B16 melanoma cells can inhibit the antigen presentation capacity of macrophages leading to reduced $CD4^+$ T cell activation [293]. Evading immune destruction and immunosuppression is a hallmark of cancer [294] and tumour cells develop strategies to regulate the immune system; mainly T

cells, DCs and NK cells. Tumour cells release exosomes and MVs which play an important role in immune escape. TEX express different immunosuppressive molecules, including checkpoint receptor ligands such as PD-L1, cytokines as TGFβ and IL-10 or ligands from the death receptor family such as FasL or TRAIL [295-297]. Furthermore, TEX can carry and transfer growth factor receptors such as EGFR (epidermal growth factor receptor) and Her-2 (human epidermal growth factor receptor 2) [279]. All these factors have been shown to be involved in immunosuppression and tumour progression.

In the peripheral blood of cancer patients the frequency of regulatory T cells ($CD4^+CD25^{high}Foxp3^+$) is elevated compared to healthy subjects [298, 299]. TEX can induce regulatory T cells *in vitro* and increase their suppressive capacity. Tumour-derived vesicles (combination of exosomes, MV and apoptotic bodies) from ascites of ovarian cancer patients and from ovarian cancer cell lines contain TGFβ and IL-10. This promotes the expansion of regulatory T cells and enhanced their suppressive capacity by upregulation the expression of CTLA-4 and FasL [300]. Other studies investigated exosomes from different malignant effusions from cancer patients and showed that also patient-derived TEX can induce regulatory T cells through the expression of TGFβ [301]. The induction of regulatory T cells leads to a more immunosuppressive tumour environment which favours progression and metastasis.

TEX can induce apoptosis in cells through the expression of FasL on the exosome surface. Melanoma cell line-derived exosomes express FasL and induce apoptosis in Jurkat cells, which might hinder lymphocytes from exerting their anti-tumour activity [296]. This might be a mechanism of the tumour to reduce the activity of cytotoxic T cells in the tumour and hence reduce anti-tumour immune responses. Similarly, plasma exosomes from oral squamous carcinoma patients express FasL, which can also induce apoptosis in Jurkat cell in a FasL dependent manner. FasL expression on exosomes correlates with the tumour stage and nodal involvement in these patients [295]. Furthermore, vesicles (combination of exosomes, MV and apoptotic bodies) from the sera from melanoma patients express the tumour antigen MAGE3/6 and FasL and inhibit $CD8^+$ T cell proliferation and induce expansion of regulatory T cells [302]. TEX can influence the tumour microenvironment by impairing differentiation of human monocytes to DCs and induction of their differentiation into HLA-DR^{low} cells, which fail to upregulate costimulatory molecules. These DCs had a suppressive activity on T cell proliferation [303].

NKG2D is an activating receptor expressed by NK cells, NKT cells, $CD8^+$ T cells and γδ T cells. Stressed cells express NKG2D ligands such as MICA and MICB, which leads to recognition by immune cells and subsequent lysis [304]. Many tumour cells release exosomes expressing NKG2D ligands. The NKG2D ligand expressing TEX can bind to immune cells and interfere with the recognition of tumour cells by NK, NKT cells etc. leading to immune escape. TEX from several cancer cell lines and from the pleural effusion of mesothelioma patients induce down-regulation of NKG2D on NK and $CD8^+$ T cells in a TGFβ dependent

mechanism, not even IL-15, a strong activator of NKG2D, could overcome this effect. [305, 306].

1.7 URINARY BLADDER CANCER

In Western countries, urinary bladder cancer has been the sixth most common cancer in men and the eighth in women. During the last years the incidence of bladder cancer cases has increased while the mortality has been decreasing. Risk factors include environmental, genetic and lifestyle factors, especially smoking [307]. 90% of all bladder cancers are urothelial cell carcinomas, only a few cases represent squamous cell carcinoma or adenocarcinoma. 70% of all bladder cancer cases/malignancies are diagnosed with non-muscle invasive bladder cancer (NMIBC) with stage T1 and T2, around 30% with highly muscle invasive bladder cancer (MIBC) (stage T3/T4) (Figure 5). Non-invasive bladder cancer has a good prognosis after transurethral resection (TUR-B) and cisplatin-based chemotherapy or BCG installation. However, the immunological mechanism underlying a favourable cancer progression of BCG is not well understood [308]. Several studies show that T cells and NK cells are important in tumour eradication. MIBC is spreading into the underlying muscle tissue and can metastasize to draining lymph nodes or even more to distant sites. Muscle invasive bladder cancer patients undergo radical cystectomy, partial removal of the tumour is not recommended due to high recurrence rates [309]. These patients have a very poor 5 year survival even with an optimal treatment. The median survival time of patients with metastatic disease is 15 months.

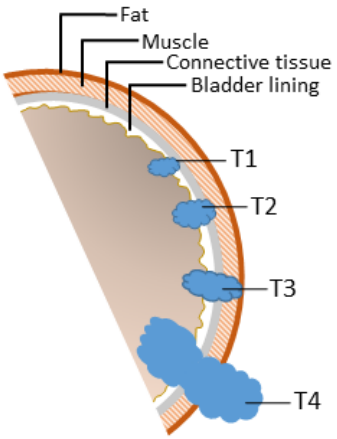

Figure 5: urinary bladder cancer stages, T1/T2 superficial bladder cancer, T3/T4 invasive bladder cancer into the connective tissue and underlying muscle

1.7.1 Exosomes in Urine from Urinary Bladder Cancer Patients

In 2004 exosomes were described for the first time in urine [138]. Urinary exosomes derived from different tissues and cells throughout the renal tract from the renal epithelia, podocytes, collecting duct to the urinary epithelia [310]. Due to the easy access urinary exosomes have been discussed as a potential marker for pathophysiological states of the urinary tract. CD24

has been described to be a marker for urinary exosomes [311]. For a long time it was believed that urinary exosomes function as a transporter of waste products instead of lysosomal degradation. However, several studies describe a role for exosomes in the urinary system [138, 311] and in regulating parts of the nephron by transmission of aquaporin 2 [312].

Several studies have been published investigating urinary exosomes from prostate cancer (PCa) and renal cell carcinoma (RCC) patients [313, 314]. Exosomes from the urine from RCC patients are enriched in proteins which are involved in cell death and cellular movement compared to healthy controls [315]. However, few studies have been investigating the role of exosomes in urinary bladder cancer (UBC) patients. Several studies compared urine from bladder cancer patients with healthy volunteers and described specific proteins enriched in the urine of cancer patients. Proteomics analysis of urinary exosomes from bladder cancer patients show upregulated expression of MUC1, CD44 and CD73 compared to healthy controls [316]. 22 proteins in urinary exosomes from bladder cancer patients were not expressed in the urine of healthy volunteers. In addition, TASCD2, a protein already described to be upregulated in different carcinomas, was found to be enriched in urinary exosomes from UBC patients [317, 318]. Another study describes the protein EDIL-3 to be highly expressed in the urine of high grade UBC patients and to induce migration and tube formation in HUVEC cell line. Exosomes from bladder cancer cell lines where EDIL-3 was knocked down were not able anymore to induce angiogenesis [318]. Urinary exosomes from UBC increase expression of mesenchymal markers like snail, a-smooth muscle actin and S100A4 and decrease epithelial markers like E-cadherin in urothelial cells [319]. In conclusion, urine provides a great source for exosomes to study mechanisms involved in cancer progression and they could serve as a source for biomarkers. However, further studies need to be conducted to define markers and to study their influence on cancer progression.

2 THE PRESENT STUDY

2.1 AIMS

Paper I To investigate if dendritic cell-derived exosomes can be loaded with the NKT cell ligand α-galactosylceramide and if these exosomes can induce NKT cell proliferation, overcome NKT cell anergy and potentiate an antigen-specific anti-tumour immune response in a B16 melanoma model.

Paper II To investigate if MHC class I molecules on exosomes are necessary to induce an antigen-specific immune response *in vivo* and if MHC mismatched exosomes can be used in cancer therapy.

Paper III To determine if dendritic cell-derived microvesicles induce a qualitatively different immune response compared to exosomes.

Paper IV To reveal the proteomic profile of urinary- and tumour- derived exosomes from patients with muscle invasive bladder cancer, to understand the metastatic process and to identify a potential marker for malignancy and prognosis.

2.2 EXPERIMENTAL SETUP

This part summarizes the most important methods used. The more detailed methods are described in each paper separately.

2.2.1 Mice

All experiments were approved by the Stockholm regional ethics committee. For the papers I – III the following mice have been used for analysis, C57Bl/6 and BALB/c mice were purchased at Taconic, $MHCI^{-/-}$ (H2Kb and H2Db gene kockout) were kindly provided by Klas Kärre, KI and bred at Karolinska Institutet, MTC. OT-I/$RAG2^{-/-}$ mice, transgenic for a TCR recognizing the SIINFEKL peptide from OVA with C57Bl/6 background; Vα14 transgenic TCR mice on C56Bl/6 background; $CD1d^{-/-}$ on C57Bl/6 background lacking NKT I and NKT II cells.

2.2.2 Bone Marrow-derived Dendritic Cell Culture

Exosomes were isolated from the supernatant of bone marrow-derived DCs from C57Bl/6, BALB/c, $CD1d^{-/-}$ and $MHCI^{-/-}$ mice. Cells were cultured at 4 x 10^5 cells/ml in RPMI 1640 (Thermo Fisher) complemented with 10 % FCS (HyClone), 1 mM sodium pyruvate, 50 mM 2-ME (Sigma-Aldrich, St. Louis, MO), 100 IU/ml penicillin/streptomycin (Thermo Fisher) 10 ng/ml IL-4 (Invitrogen), and 10% GM-CSF conditioned medium (generated from the A8653/X63 clone; a kind gift from Mattias Svensson, KI). On day 3 equal amounts of culture media were added. On day 6, ovalbumin (300 µg/ml) and/or α-galactosylceramide (100 ng/ml) were added which was washed away after overnight incubation. On day 7, cells were plated out in complete media containing 30 ng/ml LPS and 10 % exosome-depleted FCS and incubated for 2 days. On day 9, exosomes were isolated from the supernatant as described below.

2.2.3 Exosome Isolation

2.2.3.1 Exosome isolation from DC supernatant and human urine

Urine was collected during cystectomy of urinary bladder cancer patients, either directly form the ureter or before surgery from the urinary bladder. Supernatant/urine was spun at 300 x g for 10 min to remove the cells for FACS staining, at 3000 x g for 30 min to remove cell debris. After filtration through a 0.22 µm filter the supernatant was spun at 100´000 x g for 2 hours (Beckmann, LXP, Ti45 rotor) and washed once with PBS. The exosome pellet was resuspended in a small volume of PBS and frozen at -80 °C until further analysis.

2.2.3.2 Exosome isolation from tissue explants

Tumour tissue and non-tumour tissue were cut into 20 to 30 mm^3 pieces and were cultured in AimV media overnight. The next day the supernatant was spun at 3000 x g for 30 min with a subsequent filtration through a 0.22 µm filter. Exosomes were isolated by ultracentrifugation as described above.

2.2.4 Exosome Characterization

There are several standard methods to characterize exosomes and microvesicles. This part summarizes the four main methods used in the papers.

2.2.4.1 FACS

Exosomes and MVs can be analysed by FACs to determine the surface proteins. Exosomes and MVs are bound to sulfate-aldehyde latex microsphere beads (4 µm, 1.3 x 10^9 beads/ml, Invitrogen). To select only CD63 positive vesicles, 30 µg latex beads were incubated for 30 min with 30 µg anti-human CD63 antibody (clone H5C6, BD Pharmingen) following rotation overnight. After blocking with 100 mM glycine for 30 min, followed by washing with 0.5% BSA/PBS exosomes are stained with any fluorophore labelled antibody and run on any flow cytometer. This allows the analysis of one protein per staining and requires only 0.2 µg exosomes per staining.

2.2.4.2 Western blot

Certain proteins on/in vesicles cannot be detected by antibodies directly and need to be analysed by western blot. The proteins of exosomes/MVs are isolated by lysing the vesicles with RIPA buffer, sonication and vortexing. Exosomal proteins were run on Mini-Protean TGX precast gels (BioRad) and blotted to Trans-Blot Mini PVDF membranes using a Trans-Blot Turbo System (BioRad). The blots were blocked with 5% - non-fat milk in PBST overnight at 4°C. The next day the antibody of interest was used to detect the protein, together with a matching secondary antibody. The blot was visualized by enhanced chemiluminescence (GE Healthcare) with a ChemiDoc imaging system (Biorad).

2.2.4.3 Proteomics

Mass spectrometry allows the analysis of the whole exosomal proteome by using only 50 µg of total protein. In paper IV the proteomic profiles of tissue- and urine-derived exosomes were analysed by the SciLife laboratory in Uppsala. Proteins were isolated in a urea containing buffer with sonication. After trypsin digestion, peptides were purified by a Pierce C18 spin column, dried and resolved in 0.1% formic acid. Peptides were separated in a reversed-phase C18-column and subsequently electrosprayed into a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Finnigan).

2.2.4.4 Nanoparticle tracking analysis

The size distribution of a particle solution can be analysed by nanoparticle tracking analysis, nanosight (LM10HSB system, NanoSight, Amesbury, U.K.). The size of vesicles in liquid phase can be measured by the rate of light scattering and Brownian motion of the particle. The LM10 instrument allows an accurate measurement of the size distribution but delivers limited results when it comes to particle numbers in poly-dispersed samples.

2.2.5 Mouse Experiments

2.2.5.1 In vivo proliferation

40 µg of exosomes in 100 µl PBS were injected i.v. into C57Bl/6 mice on day 0. Mice were fed with BrdU (5-bromo-2'-deoxyuridine, Sigma) in drinking water (0.8 mg/ml BrdU in water supplemented with 2.5% sugar, sterile filtered 0.22 µm prior to use) to investigate *in vivo* cell proliferation. The BrdU containing drinking water was replaced every second day. On day 7, mice were sacrificed and spleen and blood was taken for further analysis. Splenocytes were stained according to the manufacture's (BD Bioscience) protocol and analysed on Fortessa LSR.

2.2.5.2 Tumour model

B16F1 melanoma cells were cultured in RPMI supplemented with 10% FCS, 100 IU/ml penicillin/streptomycin (Thermo Fisher). Cells were cultured at 2 x 10^5 cells/ml and split every second day and washed twice with PBS before injection. 30´000 or 200´000 cells were subcutaneously injected into the flank of C57Bl/6 mice and tumour volume was followed until it reached 1000 mm^3.

2.2.6 Patients

In paper IV, nine patients with invasive urothelial urinary bladder cancer (UBC) were recruited in 2014-2015. The clinical data is described in Table 4. All patients underwent primary transurethral resection of the bladder tumour (TUR-B) and clinical staging. Six patients received neoadjuvant chemotherapy (NAC) preceding radical cystectomy (RC). Three patients were diagnosed with concomitant prostate cancer.

Table 4: Patient characteristics

Patient	Preoperative clinical stage	Staging post-cystectomy	Gender	Age	NAC/noNAC	Number of Cycles	Response	Additional Information
1	cT2N0M0,G3	pT2N0M0	male	76	noNAC	0	/	
2	cT2N0M0,G3	pT0N0M0	female	69	NAC	1	CR	
3	cT2N0M0,G3	pT0N0M0	male	39	NAC	4	CR	
4	cT2N0M0,G3	pT0N0M0	male	66	NAC	3	CR	Prostatic cancer Gleason score (3+4=7)
5	cT2N0M0,G3	pT0N0M0	female	79	NAC	3	CR	
6	cT2N0M0,G3	pT0N0M0	female	77	noNAC	0	/	
7	cT2N0M0,G3	pT2bN0M0	male	65	NAC	4	SD	
8	cT2N0M0,G3	pT0N0M0	male	76	NAC	3	CR	Prostatic cancer Gleason score (3+3=6)
9	cT1N0M0,G3	pT0N0M0	male	57	noNAC	0	/	Prostatic cancer Gleason score (3+3=6)

2.3 RESULTS AND DISCUSSION

The discovery of MHC class II molecules on B cell-derived exosomes in 1996 drew interest in the immunological society for immune cell-derived exosomes. Shortly after, Zitvogel and colleagues described that immunization with DC-derived exosomes can eradicate established tumours in mice [202]. However, several phase I clinical trials showed no induction of antigen-specific immune responses after autologous immunization with DC-derived exosomes loaded with tumour peptides [219, 238]. Therefore, the understanding of how to potentiate and design exosome-based vaccines is crucial for the development of new therapeutic approaches. In study I, II and III we are investigating how DC-derived exosomes and MVs function and how they can be modified to be used as an anti-tumour treatment.

2.3.1 Co-delivery of α-Galactosylceramide and Antigen on Exosomes Induce Potent anti-Tumour Immunity (Study I)

2.3.1.1 Rationale

The induction of a potent anti-tumour immune response needs the engagement of multiple players in the immune system. We have shown that DC-derived exosomes loaded with peptides are not sufficient for inducing antigen-specific immune responses *in vivo*. However, loading of the whole antigen onto exosomes led to activation of $CD4^+$ [320] and $CD8^+$ T cells [203] *in vivo*. To further potentiate the immune response we loaded the NKT cell ligand αGC onto DC-derived exosomes and investigated whether T and B cell responses were amplified.

αGC has drawn interest during the last years as a potent stimulator of NKT cells, followed by the production of a wide range of cytokines [107, 108], maturation and licensing of DCs and subsequent $CD8^+$ T cell activation [321]. αGC induces anti-tumour immunity mainly through the secretion of IFNγ by NKT and NK cells and at later time points by production of IL-12 by DCs [119]. However, injection of soluble αGC into patients with solid tumours induced a transient increased cytokine production but clinical results were limited [134]. This might be explained by the low NKT cell numbers in cancer patients [322], exhaustion of NKT cells after several injections [112] and by suboptimal delivery to DCs. The uncontrolled uptake of free αGC by different cell types can be overcome by using a specific delivery system such as cells or nanoparticles. αGC-loaded DCs induce a better NKT cell response compared to soluble αGC alone [113, 323]. Similar results were seen when coupling αGC to nanoparticles [324]. In addition, antibody mediated targeting of αGC-loaded nanoparticles to DEC205 on DCs led to an increased NKT cell activation and prevented their hyporesponsiveness compared to non-targeted nanoparticles [325]. In conclusion, coupling of αGC onto nanoparticles induces better effects *in vivo*. Therefore, loading of CD1d-expressing exosomes with αGC would be a possibility to induce a more efficient anti-tumour response.

2.3.1.2 αGC on exosomes activate NKT cells and potentiate adaptive immunity

To investigate whether exosomes could be loaded with αGC, we first examined if exosomes from αGC-loaded DCs were able to activate NKT cell *in vitro*. We prepared exosomes from

BMDCs pulsed with αGC (Exo-αGC) or OVA antigen (Exo-OVA). Exo-αGC induced proliferation of Vα14 splenocytes (genetically modified to express only the Vα14-Jα18 TCR) and induced increased the numbers of IL-4 producing splenocytes measured by ELISPOT, no response was seen after Exo-OVA stimulation. In contrast, exosomes derived from BMDCs lacking the CD1d molecule ($CD1d^{-/-}$ Exo-αGC) induced less proliferation of NKT cells and less IL-4 producing splenocytes. This indicates that αGC is mostly but not exclusively bound to CD1d on exosomes and might be integrated into the exosome membrane or into the exosome lumen. Similar results were obtained *in vivo* in B6 mice. We injected 40 µg exosomes i.v. and fed the mice with BrdU to assess *in vivo* proliferation. NKT cells strongly proliferated in response to Exo(αGC-OVA) within the first three days and expressed high levels of IFNγ until day 5. No response was detected upon Exo-OVA or PBS injection.

Interestingly, we also detected activation of NK cells already 1 day after Exo(αGC-OVA) injection. It has been shown that activation of NKT cells by αGC also leads to activation of NK cells already 90 min after αGC exposure mediated through IFNγ [110]. NK cells are important players for tumour killing and immune surveillance. They recognize, according to the "missing-self" theory, tumour cells if they downregulate MHC class I expression.

The induction of tumour-specific T cells and tumour-specific antibodies is a major goal of cancer immunotherapy. However, strong stimuli are needed to overcome the immunosuppressive microenvironment in cancer patients. In study I we detected an amplified induction of OVA-specific $CD8^+$ T cells 7 days after injection, which was dependent on αGC and OVA on exosomes. Injection of exosomes loaded with αGC and SIINFEKL did not induce antigen-specific T cells. Therefore, we can conclude that αGC does not override the dependency on whole OVA, and does not induce a polyclonal expansion of $CD8^+$ T cells. The activation of OVA-specific $CD8^+$ T cells was dependent on CD1d on exosomes. Injection of $CD1d^{-/-}$ Exo(αGC-OVA) showed significantly lower induction of SIINFEKL-specific cells.

Antibody-dependent cell cytotoxicity (ADCC) is an important mechanism in anti-tumour immunity. ADCC occurs when tumour-specific antibodies bind to the tumour cells and can be recognized by Fc receptors on immune cells such as NK cells, macrophages and neutrophils. The activation through the Fc receptor leads to release of perforin and granzyme and to destruction of the tumour cell membrane and finally to apoptosis. Interestingly, antibodies used in the clinic such as rituximab (anti-CD20 antibody), trastuzumab (anti-HER/neu antibody) and cetuximab (anti-EGFR antibody) have been shown to mediate ADCC [326]. Therefore, we were interested if exosomes loaded with αGC induce a potent antigen-specific humoral response. Exo(αGC-OVA) induced strong proliferation of $CD4^+$ T cells, which was dependent on iNKT cells *in vivo* since $CD4^+$ T cell proliferation was abrogated in $CD1d^{-/-}$ mice. In addition, germinal center responses, including T follicular helper cells and germinal center B cells, were potentiated after Exo(αGC-OVA) injection compared to Exo-OVA or PBS. Interestingly, Th1 promoting IgG2c antibodies and OVA-specific IgG antibodies were increased after Exo(αGC-OVA) injection, whereas levels of total IgG and IgG1 were similar. Induction of Th1 immunity is crucial for a potent anti-tumour immune response. We conclude that αGC on exosomes boost the immune response

and induce specific B cell responses by activation of $CD4^+$ helper T cells. In general, the advantage of using αGC on exosomes as an adjuvant is to induce a broad activation of different immune cells orchestrating a potent anti-tumour response.

2.3.1.3 Exosome-associated αGC and OVA are more potent that soluble αGC and OVA in inducing adaptive immunity

DC licensing through antigen-specific $CD4^+$ T cells and the presence of TLR ligands is an important step for the induction of cross-presentation [327]. Nevertheless, also NKT cells can provide help for DC priming. Licensing of DCs through NKT cells requires presentation of the protein antigen and the glycolipid on the same DC [321]. Therefore, αGC and OVA loaded nanoparticles, and in particular exosomes, are likely superior to soluble antigens in delivering the protein antigen and the glycolipid on the same particle. We show that soluble αGC and OVA are potent stimulators of NKT cells, but are inducing less potent adaptive responses. γδ T cell, OVA-specific $CD8^+$ T cell and $CD4^+$ T cell proliferation was augmented after Exo(αGC-OVA) injection compared to soluble antigen (Figure 6). Furthermore, induction of germinal centers and OVA-specific antibody production was strongly enhanced after the second exosome injection. Thus, exosomes loaded with αGC and OVA can deliver strong signals directly to the same DC, which enables induction of licensed DCs and enables better cross-presentation.

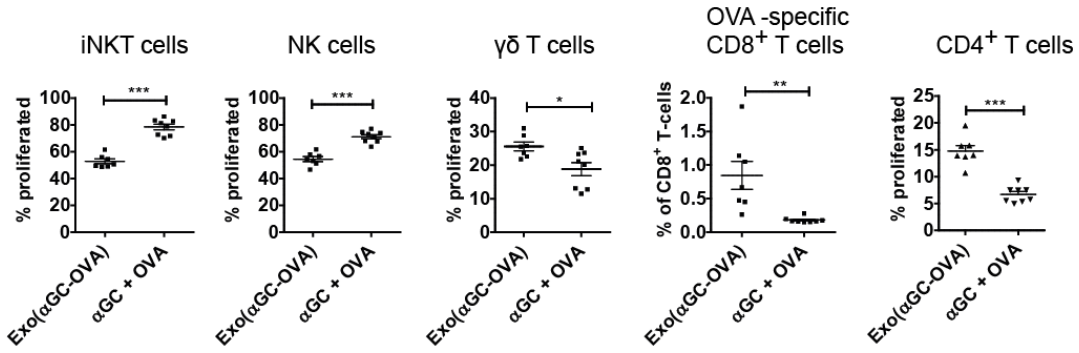

Figure 6: Exosome-bound αGC and OVA are more potent in inducing adaptive immunity compared to soluble αGC and OVA. Enhanced proliferation of iNKT and NK cells can be detected after soluble OVA and αGC injection. In contrast, γδ T cells, OVA-specific $CD8^+$ T cells, $CD4^+$ T cell responses are enhanced after Exo(αGC-OVA) treatment and diminished after soluble αGC and OVA injection, data is published in paper I

2.3.1.4 Exo(αGC-OVA) administration does not induce anergic NKT cells and prolongs survival significantly in a B16 tumour model

As already discussed previously, repeated injections of soluble αGC induces anergic NKT cells. Therefore, we wanted to investigate if two injections with Exo(αGC-OVA) (at day 0 and day 14) leads to unresponsive NKT cells. Measurement of serum IFNγ levels one day after the second injection (d15) revealed higher IFNγ levels in the Exo(αGC-OVA) injected group compared to the group injected with soluble αGC and OVA alone. Hence, NKT cells are still able to produce cytokines after the second Exo(αGC-OVA) injection and are not

anergic. Furthermore, restimulation of splenocytes with αGC at day 21 showed more INFγ producing cells after Exo(αGC-OVA) injection compared to soluble antigen. In addition, glycolipid and SIINFEKL-specific T cells were more abundant. Two injections of Exo(αGC-OVA) also increased percentages of germinal center B cells and antigen-specific IgG levels dramatically. This further demonstrates that exosome-associated αGC allows several injections and boosts the anti-tumour immune response.

Finally, we were interested to know whether boosting of adaptive immunity by exosomes led to a prolonged survival in a B16/OVA model. Both one and two injections of Exo(αGC-OVA) prolonged the survival significantly and led to a greater increase of tumour-infiltrating OVA-specific T cells and OVA specific IgG levels in the serum compared to soluble αGC and OVA alone (Figure 7).

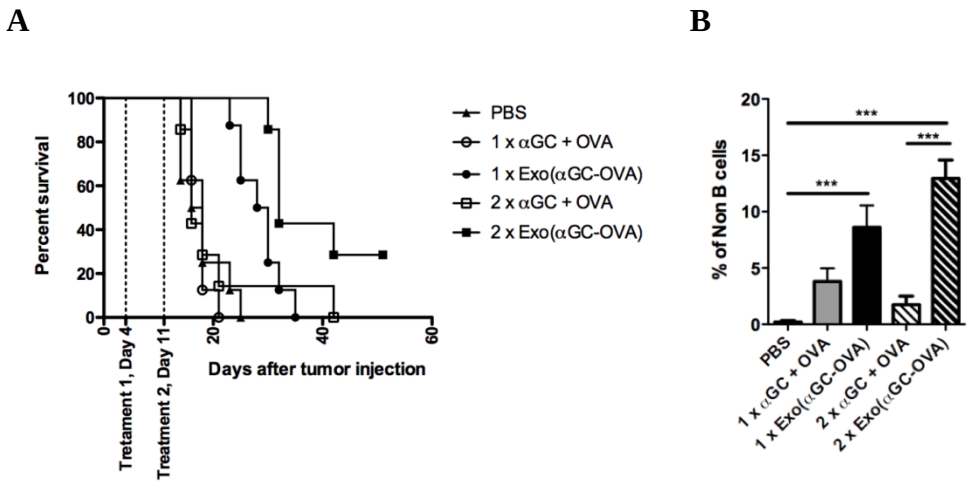

Figure 7: Exo(αGC-OVA) prolonged survival and induced more infiltrating antigen-specific T cells, mice were injected with 1 x 10^5 B16/OVA cells s.c. and treated with 40 µg exosomes on day 4 (1x injection) or day 4 and 11 (2x injections), mice were sacrificed when the tumour reached a volume of 1,000 mm^3 A) Kaplan-Meier survival curve, B) quantitation of OVA-specific $CD8^+$ T cell infiltration into the tumour, data is published in paper I

2.3.1.5 Outlook Study I

Our findings show that exosome-associated αGC and OVA do not induce anergic NKT cells and are superior in inducing an adaptive immune response compared to soluble αGC and OVA. However, the response might be even stronger if exosomes are directly targeted to the DC. As already mentioned above, DCs can also be licensed by NKT cells and co-delivery of the glycolipid and the protein antigen to the same DC could enhance activation of $CD8^+$ T cells. Therefore, mechanism of DC targeting by exosomes also need to be investigated to potentiate exosomal cancer vaccines.

Furthermore, when designing exosomal vaccines for therapy one also needs to take into consideration that the NKT cell levels in humans are reduced around 10 % compared to mice and that the variability in the numbers of NKT cells between patients is tremendous. Only a

subgroup of patients might respond to NKT cell mediated therapies, therefore selection of the right patient group is important for future therapeutic approaches.

2.3.2 MHC Independency of Exosomal Vaccines (Study II)

B cell- and DC-derived exosomes carry MHC molecules on the surface and are able to stimulate T cells directly. However, many of these studies used T cell lines *in vitro* and never non-primed cells *in vivo* [146, 206]. In addition, the presence of DCs *in vitro* greatly enhances T cell proliferation [207]. Hence, we questioned the importance of MHC molecules on exosomes to stimulate T cells and their direct stimulatory capacity *in vivo*. Several clinical trials using peptide-loaded DC-derived exosomes as anti-cancer vaccines showed limited effects; induction of antigen-specific $CD8^+$ T cells was not detectable and clinical outcome was limited [219, 237, 238]. Furthermore, the use of the patient´s own monocyte-derived DCs might not be feasible due to clinical status and immunosuppression. Thus, allogeneic exosomes loaded with innate stimuli and antigens might be another possibility in cancer therapy.

In study II we were interested in investigating if MHC molecules are needed to induce an antigen-specific T cell response *in vivo* and furthermore, if MHC mismatch leads to equal anti-tumour immunity in a B16 melanoma model. This would improve the feasibility of using exosomes as a vaccine and opens up new possibilities of using MHC mismatched parent cells as exosome producers. We isolated exosomes from BMDC from C57Bl/6 WT mice, $MHCI^{-/-}$ mice (C57Bl/6 background) and from BALB/c mice. First, we demonstrated that all exosomes shared phenotypic characteristics and expressed similar levels of costimulatory molecules such as CD40, CD80, CD86 and tetraspanins such as CD9, CD63 and CD81. In addition, CD1d expression on exosomes and OVA concentration were comparable on all exosomes types. Thus, we conclude that all exosomes are phenotypically similar but differ in their expression of MHC molecules.

Intravenous injection of WT and $MHCI^{-/-}$ exosomes either loaded with OVA and αGC or OVA alone induced similar levels of OVA-specific $CD8^+$ T cells in the spleen. Thus, we conclude that peptide/MHC complexes on exosomes are negligible to induce an adaptive immune response if the whole protein antigen is present. Furthermore, to test the feasibility of MHC mismatched exosomes in a tumour model we used exosomes from BALB/c BMDC. Here, both MHC class I and II are different from C57Bl/6 mice. Exosomes used in a clinical setting might not derive from the patient´s own cells and a MHC mismatch might occur and could even be beneficial. Indeed, injection of BALB/c exosomes led to comparable levels of OVA-specific $CD8^+$ T cells in the tumour and OVA-specific IgG levels in the serum of these mice. Hence, MHC molecules present on DC-derived exosome are not necessary for the induction of an anti-tumour immune response.

Different mechanisms of how DC-derived exosomes can stimulate an immune response have been discussed. MHC/peptide complexes on exosomes could directly bind and activate T cells (Figure 8A). However, the proliferation of T cells in the presence of exosomes only is

low and can be amplified by DC [207]. DCs could also take up exosomes, degrade the proteins and load the antigen on newly synthesized MHC molecules (Figure 8B). The third alternative is the recycling of exosomal MHC/peptide complexes and the presentation of the foreign MHC on the DC surface (Figure 8C) [209]. Study II demonstrates that mechanism B is most important *in vivo* (in our system).

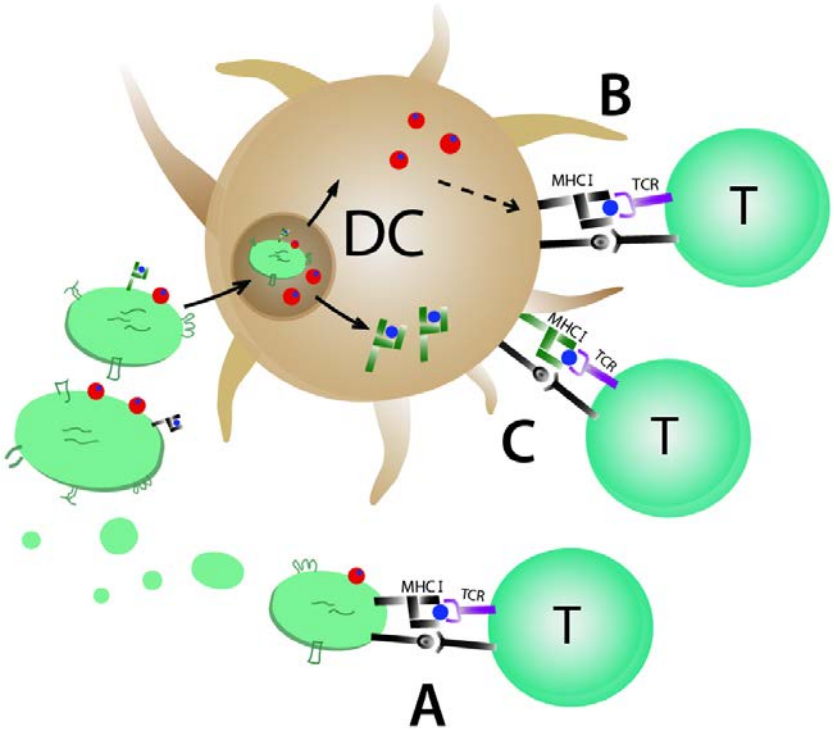

Figure 8: Different pathways of how exosomes could activate T cells, A) direct simulation through MHC/peptide complex, B) Degradation of exosome content and new loading of DC-derived MHC molecules, C) recycling of MHC/peptide complex from the exosomes to the DC surface.

2.3.2.1 Outlook Study II

We have shown that exosomes for cancer therapy do not need to be autologous. In contrast, allogeneic MHC molecules could even add to the adjuvant effect of the DC-derived exosomes. Therefore, the use of allogeneic exosomes in the clinic could be favourable. Interestingly, our preliminary results show that repeated injections of MHC mismatched exosomes led to a stronger immune response compared to injection of syngeneic exosomes (unpublished observations). The underlying mechanism of this effect and if allogeneic exosomes induce anti-MHC antibodies needs to be investigated.

2.3.3 Exosomes and Microvesicles Induce Qualitatively Different Immune Responses (Study III)

In paper III we compared the immunostimulatory capacity of MV and exosomes isolated from the same cell side by side *in vivo*. To this date, MVs have not been extensively studied in vaccine settings. Therefore, we wanted to investigate if MVs and exosomes from the same DC induce qualitatively different immune response *in vivo*.

Electron microscopy pictures of exosomes and MVs from preliminary experiments showed a clear contamination of the MV pellet with small vesicles. Therefore, we decided to purify the MV pellet with anti-tetraspanin beads to remove the small vesicles. Purification with anti-CD63 and anti-CD81 coated beads did not show good purification results, while anti-CD9 beads showed a reduced number of small vesicles measure by NTA. The shift in the particle diameter measured by NTA showed a successful removal of exosome-like vesicles expressing tetraspanins CD9, CD63 and CD81 measured by FACS after anti-CD9 bead purification.

We show that MV and exosomes share phenotypic characteristics by FACS and expressed similar levels of CD9, CD54, CD80, CD86, MHC class I and MHC class II. However, CD63 levels were lower in MVs compared to exosomes. In addition, phosphatidylserine expression measured by annexin V binding was higher in exosomes, however not significantly different to MVs.

It has been shown that exosomes can be taken up via lipid rafts and by binding to annexins expressed on cells [328]. This could account for different biodistribution and uptake or clearance by different cell types. MVs and exosomes might also differ in their RNA content. This could be of importance since it has been shown that miRNA in exosomes from cancer cells can bind TLR7 from macrophages and induce expression of cytokines [271]. Their phenotypic differences and the influence on biodistribution, uptake and immune activation need further investigation.

To investigate the *in vivo* stimulatory capacity of exosomes and MVs, we injected 40 μg of vesicles intravenously into C57Bl/6 mice and analysed the immune response 7 days after injection. Exosomes were superior in inducing antigen-specific $CD8^+$ T cells compared to MVs measured by H-2Kb-pentamers and ELISPOT restimulation with SIINFEKL peptide. In contrast, MV induced significantly more plasma cells. However, both vesicle types induced similar levels of IgM antibodies in the serum, whereas only exosomes induced IgG2c and OVA-specific IgG antibodies. No differences were seen in total IgG and IgG1 levels. Hence, exosomes induced a Th1 biased immune response, similarly to what we have seen in paper I, whereas MV rather induced an unspecific activation.

MV and exosomes might also differ in their biodistribution. Intravenously injected MV and exosomes will probably be captured in the marginal zone of the spleen. Depending on the size, surface charge and surface proteins they will be taken up and processed differently. DCs bind preferentially 100 nm sized particles [329]. Therefore, MV might be captured by

macrophages, which clear them from the blood circulation without inducing a potent immune response. Macrophages have been described to be important cells in the marginal zone to capture extracellular vesicles [163]. In contrast, exosomes might be taken up by DCs to a larger extent.

Since exosomes were more potent in activating an antigen-specific immune response we were interested if both vesicle types carry similar amounts of OVA. Analysis by ELISA and WB revealed a much higher concentration of OVA in exosomes compared to MVs, both on the surface and inside. Therefore MVs might activate the immune system unspecifically and are not able to induce antigen-specific immune responses due to the low amount of antigen. The MR expressed on BMDC (discussed later) could influence the uptake and intracellular fate of OVA and the location in endosomes.

2.3.4 Discussion Paper I - III

2.3.4.1 Exosomes – Natural Liposomes

Protein-based vaccines require an adjuvant to activate the immune system. Nanoparticle- and liposome-based vaccines can carry innate stimuli such as TLR ligands to activate DCs efficiently, leading to a potent immune response. At the same time, they are delivery vehicles, which can carry and transfer antigens. Exosomes can function as natural adjuvants and deliver innate stimuli and activate the innate immune response. In study I, we show that loading of αGC leads to efficient activation of NKT cells and to enhanced adaptive immunity. Interestingly, exosomes can transfer proteins and antigens to immune cells. The location of the antigen can be important for their immunogenicity. Exosomes can carry the antigen on the surface or inside, but only antigens on the surface are able to stimulate B cells directly. Exosomes used in study I, II and III carry OVA on the surface and might be able to activate OVA-specific B cells directly. Nevertheless, antigen delivery by exosomes might overcome the dilution of the antigen *in vivo* and enables the delivery of low amounts of antigen to antigen presenting cells.

The lipid composition of exosomes is similar to cells. They express certain integrins and tetraspanins, which have been shown to be important in their biodistribution and cell uptake. As a natural vesicle, exosomes are negatively charged. In study III we show that exosomes and MV express negatively charged PS on their surface. In contrast, several studies have been using positively charged liposomes as vaccine adjuvants to enhance the interaction between cells and liposomes [330]. However, PS on liposomes has a potent adjuvant effect and PS positive liposomes are preferentially taken up by conventional DC [331]. In conclusion, exosomes express proteins and lipids, which are important for their uptake and can promote similar functions as liposomes.

2.3.4.2 BMDC-derived Exosomes and the Uptake of OVA

In study I,II and III we used BMDC-derived exosomes loaded with OVA antigen. OVA was added to the culture at day 6 and incubated overnight. The next day cells were washed and the free OVA was removed and cells were cultured for two days in exosome-depleted media. The mechanism of the OVA loading process onto exosomes in DCs is unknown and the reason for the lower loading of OVA onto MVs also needs further studies.

More is known about how free OVA is taken up by cells. In general, DCs take up soluble antigens through two different mechanisms, micropinocytosis or clathrin-mediated endocytosis. Macropinocytosis is a non-specific mechanism and mediates uptake through membrane ruffles [332]. In contrast, clathrin-mediated endocytosis is an active mechanism, which depends on cell surface receptors such as Fc receptors or the mannan receptors (a C-type lectin receptor, MR, CD206) [333]. The uptake of OVA is mainly mediated via clathrin-mediated endocytosis through the MR and to a lesser extent through macropinocytosis [334, 335]. Interestingly, depending on the route of uptake, OVA peptides are preferentially loaded onto MHC class I or MHC class II molecules. Uptake of OVA via the MR leads to more cross-presentation, while uptake via macropinocytosis to more MHC class II loading. Importantly, mainly antigen which is taken up via the MR is found in early endosomes and can be cross-presented. This is not the case if it is taken up via scavenger receptors. Interestingly, only $CD8^+$ DC express the MR [334, 335]. Hence, depending on the uptake mechanism, OVA is shuttled to different endosomal compartments. In our system, the uptake of OVA via the MR receptor might lead to exosome loading. We detect a great amount of OVA inside of exosomes (based on western blot results), thus, OVA needs to be released into the cytosol from the endosome to be loaded into the exosome lumen. Uptake through the MR leads to more cross-presentation and therefore to more OVA in the cytosol. However, in study III we did not detect OVA inside of MV. The process behind this discrepancy is still unclear and needs further investigation.

OVA on the surface of our exosomes can also influence their biodistribution and uptake through the MR *in vivo* at the marginal zone. Interestingly, peptides and proteins conjugated to mannan stimulate $CD4^+$ T cells much better compared to non-conjugated antigens [336]. Several clinical trials using MUC1 bound to mannan for DC- targeting showed promising results and the induction of humoral and cellular immune responses [337, 338]. Therefore, the importance of the MR receptor on the biodistribution and uptake of our OVA loaded exosome vaccines needs to be determined and compared with other antigens.

2.3.4.3 General Problem of Tumour Vaccines

For designing new tumour vaccines the knowledge of the tumour antigen is crucial. Very often, tumour antigens are endogenous proteins. Therefore, the presence of T cells recognizing these antigens seems impossible due to central tolerance and the deletion of self-reacting T cells in the thymus. However, certain T cells escape central tolerance due to low expression of mainly carcinoembryonic and gamete antigens (major tumour antigens) in the

thymus. Unlike positively selected T cells, these tumour antigen-specific T cells express a low affinity TCR since high affinity clones are deleted in the thymus. In contrast, a vaccine against a virus activates virus-specific T cells in the periphery, which have not been selected in the thymus due to the foreign nature of the protein. Thus, it is difficult to design exosome-based cancer vaccines and to induce a potent immune response by using neoantigens expressed by the tumour. Furthermore, some tumours have a low mutation rate and therefore express low levels of neoantigens and are therefore not easily recognizable by the immune system. In addition, the use of self-antigens might need an even more personalized approach to detect specific neoantigens in the patients. In study I, II and III we have used OVA as a model for tumour antigen loaded onto exosomes. OVA can be used in very high concentrations which might not be feasible for clinical applications. Therefore, new antigen delivery methods for exosome loading need to be developed.

Another problem when using tumour vaccines is the presence of regulatory T cells, MDSC and tumour-associated macrophages (TAM) in the tumour and high levels of immune suppressive cytokines like IL-10 and TGFβ in the tumour. In addition, TILs express high levels of checkpoint molecules which lead to immune escape. Therefore, targeting immune suppressive factors in combination with a vaccine might be a successful approach to overcome the immune suppressive microenvironment in the tumour. Several approaches are currently under investigation. Targeting immune checkpoints like CTLA-4 (ipilimumab) with monoclonal antibodies showed good clinical results in melanoma patients [61]. Targeting of Tregs with an IL-2-diphteria toxin conjugate led to a 16 fold increase in tumour-specific cytotoxic T cells in renal cell carcinoma patients [339] and COX-2 inhibitors suppressed MDSC and increased TILs in a mouse glioma model [340]. Targeting the microenvironment and immune suppressive cells in the tumour in combination with tumour vaccines might be successful. However, the balance between induction of a strong anti-tumour immune response and the development of autoimmunity needs to be considered.

2.3.4.4 Future Perspectives – Exosomal Vaccines

Cancer immunotherapy currently relies on strategies such as antibody-based therapies, cytokines and adoptive cell transfer. Exosome-based cancer therapy has shown limited results in clinical trials. However, in the last years the understanding of how to manipulate and engineer exosomes has increased rapidly.

One of the major problems in designing exosomal vaccines is the delivery of antigens on or in exosomes. Since it has been shown that peptide loaded exosomes are not efficient in inducing specific immune responses *in vivo*, the loading of whole protein antigens needs to be further investigated. In paper I, II and III we added OVA to the BMDC culture to be loaded on exosomes. The disadvantage of this method is the huge protein amount needed. Therefore, new antigen loading methods needs to be developed to increase the feasibility of exosome-based cancer vaccines. Antigen can be genetically coupled to tetraspanins to be exposed on the exosome surface. However, this method requires a stable transfection of a cell line, which secretes immunostimulatory exosomes. Until this day, it is not fully understood why certain

exosomes are immunostimulatory and others not. Interestingly, Hao and colleagues showed that DC-derived exosomes are superior in stimulating $CD8^+$ T cells *in vivo* compared to tumour-derived exosomes loaded with the same antigen [230]. In paper II we show that the induction of an immune response is independent of MHC molecules if the whole antigen is present. Therefore, exosomes could possibly be isolated from engineered cell lines without the need for the patient's own cells. This would also allow isolation of exosomes from large-scale bioreactors, which would increase the particle numbers greatly. However, the understanding of the molecules needed to induce an immune response and the specific targeting of cells needs further investigation.

$CD169^+$ macrophages, subcapsular sinus macrophages in the spleen, capture B cell-derived exosomes. $CD169^{-/-}$ mice have a diminished immune response against antigen-pulsed exosomes [163]. In the tumour draining lymph nodes they clear and limit dissemination of tumour-derived vesicles [214]. Macrophages in lymphoid organs might limit the immunostimulatory function of DC-derived exosomes. Therefore, exosome targeting to specific immune cells or blocking of specific receptors on macrophages might induce more potent responses. Hence, the understanding to which cells and receptors exosomes naturally bind and how they are taken up would increase the knowledge for improving exosomal vaccine design.

2.3.5 Exosomes from the Tumour Site Express a Malignant Memory Phenotype (Study IV)

In paper IV we wanted to investigate the metastatic process induced by exosomes from urine and from the tumour site of urinary bladder cancer patients and if they express markers for diagnosis and recurrence.

First, we needed to establish a protocol for exosome isolation from human tumour tissue. Tumour-derived exosomes were isolated from tissue explants from the tumour site and from healthy bladder tissue removed during cystectomy cultured for 24 hours in media. Exosomes released during this time were collected and analysed. Furthermore, we isolated exosomes from urine coming directly from the ureter and from the urinary bladder, collected during surgery. All patients underwent a TUR-B before cystectomy, a cytoscopic surgery where the tumour, or parts of the tumour, are removed from the bladder. We show that exosomes from the urine and from the tumour site express malignant phenotypes, even when no macroscopic tumour is left at the previous tumour site after TUR-B. We detect a separation of the urine whether it has been in contact with the tumour or not. We propose that exosomes express a metastatic memory in the tissue and these conditions might influence recurrence of macroscopic tumours.

Tissue-derived exosomes separated according to malignancy and in dimension 1 correlated with gender, prostate cancer and number of NAC cycles. We could define 487 proteins in urine from the bladder and 30 from the tumour site, which are specific predictors of malignancy. Bladder urine and tumour site exosomes shared 6 proteins (ATP6V1A, Rab5C,

GNAS, BASP1, GNA13, HLA-DRB1). These 6 proteins have been described to be involved in cancer progression. Interestingly, even if no tumour is left the tissue- and urine-derived exosomes express a carcinogenic phenotype which might be involved in the formation of pre-metastatic niche. They might derive from microscopic tumours or from epithelial cells affected by the tumour cells. ATP6V1A, an ATP-dependent proton pump, mediates acidification of intracellular organelles and it is expressed in the plasma membrane of invasive tumour cells and can mediate metastasis [341]. Rab5C, involved in fusion processes of intracellular vesicles, involved in metastasis and regulated EGFR activation [342]. GNAS and GNA13, proteins from the G-protein family, are signalling proteins and can express mutations in several malignancies.

The proteins correlating with malignancy were involved in different pathways including metabolism, platelet activation, growth factors and the semaphoring pathway. Due to the high proliferation rate, cancer cells have a higher need for nutrients; they require rapid generation of ATP, increased synthesis of macromolecules and control of the redox status. However, it is not clear if these factors apply also for slow growing tumours in humans, since many studies have been examining *in vitro* cell cultures.

One major metabolic phenotype of cancer cells is the shift from oxidative phosphorylation to glycolysis and the breakdown of glucose to lactate in an oxygen-independent manner. Therefore, the demand of glucose is much higher in cancer cells compared to normal cells, since glycolysis leads to production of less ATP compared to oxidative phosphorylation [343]. Production of lactate also supports the acid microenvironment seen in cancerous tissue. The enhanced metabolism also leads to production of NADPH, which is needed to prevent reactive oxygen species induced damage [344].

A second pathway involved was platelet activation. Platelets are secreted by megacaryocytes in the bone marrow. Their main function is to halt bleeding after injury. However, cancer patients often suffer from thrombosis and often very severely if they are diagnosed with metastatic disease [345]. Activated platelets can interact with circulating tumour cells and facilitate adhesion to the endothelium and subsequently extravasation and metastasis [346]. Several studies showed that platelet-derived microvesicles stimulate cell proliferation and matrix metalloprotease (MMP) expression, increased the adhesion to the endothelium and promoted metastasis *in vivo* [273, 347].

Semaphorins are a very diverse group of proteins and were first described to direct neuronal axons to the right target. More recently they have been described to either promote tumour progression or to function as tumour suppressors. They can affect tumour progression in different ways by promoting angiogenesis, recruiting bone marrow cells or directly targeting adhesion and cytoskeletal rearrangement of tumour cells, thus influencing cell attachment and migration [348]. The most studied semaphoring in cancer metastasis is semaphoring 4D (SEMAD4D) which can mediate angiogenesis and tumour progression in head and neck squamous cell carcinoma. Furthermore, it is highly expressed in transformed epithelial cells but not in non-invasive cells [349]. Exosomes from malignant bladder tissue

and bladder urine might contribute to tumour progression by activating the semaphorin pathway.

Certain proteins were highly upregulated in the urine from the bladder compared to the urine from the ureter. Proteins which were already described in malignancies and could function as prognostic markers are: transmembrane protease serine 2 (TMPRSS2), acid phosphatase, prostate (ACPP) and phosphoglycerate mutase 1 (PGAM1). TMPRSS2:ERG fusion protein was described to be shed in prostasomes [350, 351]. ACPP was increased in the serum of prostate cancer patients and have been described to be associated with vesicle structures [352, 353]. PGAM1 expression in tissue has been described to correlate with the clinical status of the patient [354].

Many proteins upregulated in urine and tissue might reflect an ongoing inflammation after TUR-B. Several complement proteins were upregulated in the bladder urine and proteins associated with eosinophils were upregulated in tumour site tissue. It was demonstrated that eosinophils infiltrate into the bladder tissue after TUR-B [355]. Therefore, Charcot-Lyden crystal galectin (CLC) and eosinophil peroxidase (EPX) expression in exosomes from the tumour site might reflect cell infiltration after surgery. Furthermore, carboxypeptidase M, a marker for macrophage maturation and mainly expressed by M2 macrophages could also reflect the ongoing inflammation in the tissue or the accumulation of tumour-associated macrophages. Interestingly, this protein has never been described in urine from UBC patients.

In this study we have shown that exosomes can be distinguished according to their proteomic profile if they are coming from the ureter or have been in contact with the tumour site, or if they were isolated from the previous tumour site of from healthy bladder tissue. Furthermore, patients with concomitant prostate cancer express a specific exosomal phenotype in the bladder exosomes. We conclude that even when no tumour is left exosomes express a malignant memory phenotype which might be involved in recurrence and progression.

Our unveiling of malignant memory in the tissue exposed to the tumour and even in urine, suggests that the tissue is altered and prepare to harbour new tumour cells. The fact that this is detected in the exosomes from the tissue, suggests that exosomes play a role in this process and might contribute to metastasis formation at distant sites. Further understanding of this process might give us the tools to reverse or inhibit the process of relapse and metastasis.

2.3.5.1 Future Perspective - Tumour-derived Exosomes

Tumour-derived exosomes have been shown to be involved in different processes in tumour progression. They influence the tumour microenvironment, promote metastasis in distant lymph nodes and organs, transfer oncogenic proteins, breakdown extracellular matrix proteins and induce and immune suppressive environment.

Invasion and metastasis are the main treatment failures and connected with poor prognosis. The understanding of how exosomes and microvesicles promote metastasis and help inducing the pre-metastatic niche at distant sites could help developing new therapeutics. TEX mediate organ-specific metastasis through expression of specific integrins and induce changes in gene expression in distant organs [272]. However, the mechanism behind the metastasis promoting effect is not known and whether specific receptors on endothelial cells or immune cells take up exosomes specifically needs to be determined.

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